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PHARMACOLOGICAL EVALUATION OF NOVEL CALCIUM CHANNEL ANTAGONISTS AND AGONISTS

by

M.C LI-KWONG-KEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

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FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA

SPRING 1986

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Supervisor

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Date April 17, 1986

DEDICATED

TO

MY FAMILY

ABSTRACT

The pharmacological activities of 76 novel dihydropyridine analogs of nifedipine were evaluated on smooth and/or cardiac muscle, in an attempt to further investigate the structure activity relationships (SAR) of this group of calcium channel antagonists. The 76 novel dihydropyridine analogs were divided into 5 series, and SAR were derived for each group of compounds. With respect to the 1,4-dihydropyridine ring of nifedipine and Bay K8644, the 5 series of compounds were: (1) C4-pyridinyl analogs of nifedipine with substituent variations at the C3 and C5 positions (2) C4-dihydropyridinyl derivatives of nifedipine with substituent variations at the C3 and C5 positions (3) C4-tetrahydropyridinyl analogs of nifedipine with substituent variations at the C3 and C5 positions (4) Analogs of nifedipine with the 1,4-dihydropyridine ring replaced by a 1,2-dihydropyridine (5) C4-pyridinyl analogs of Bay K8644. In general, the novel calcium channel blockers showed the following SAR: (a) Replacement of the C4-o-nitrophenyl ring of nifedipine by pyridinyl, dihydropyridinyl and tetrahydropyridinyl reduced activity (b) At the C4 position, the relative order of activity was 2'- > 3'- > 4'-pyridinyl and 3'- > 4'-dihydropyridinyl (c) Activity was enhanced when the size of the alkoxycarbonyl substituents at the C3 and C5 positions were increased (d) Non-identical substituents at the C3 and C5 positions produced compounds of better activity than identical ones (e) Introduction of CN or

N,N-dimethylaminoethoxycarbonyl at either C3 and/or C5 positions decreased activity (f) SAR derived for the 1,2-dihydropyridine series of compounds were significantly different from those obtained in the 1,4-dihydropyridine series (g) Methylation at the N1 of the 1,4-dihydropyridine ring of nifedipine reduced activity (h) Planarity of the C4-ring substituent was important for activity (i) Perpendicular orientation of the C4-ring substituent to the 1,4-dihydropyridine ring of nifedipine appeared to represent the ideal conformation for good calcium antagonist activity. The novel calcium channel blockers had activities in the range of 10^{-9}M to 10^{-6}M, as compared to nifedipine which was active at 10^{-8}M. One of the novel calcium channel blockers was shown to possess antihypertensive action on conscious SHR rats. In addition, C4-dihydropyridinyl analogs with bulky ester substituents at both C3 and C5 positions appeared to have a longer duration of action. The C4-3'- and C4-4'-pyridinyl derivatives of Bay K8644 exhibited calcium agonist properties whereas the 2'-pyridinyl analog had calcium antagonist activity. Correlations of the pharmacological activities of the novel calcium channel blockers with their corresponding ability to displace [^{3}H]-nitrendipine from its binding sites suggest that these compounds mediate their pharmacological effects through the same dihydropyridine receptors.

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Table of Contents

Chapter		Page
1.	LITERATURE SURVEY	1
	1.1 CALCIUM AND MUSCLE CONTRACTION	1
	1.1.1 Regulatory Role of Intracellular Calcium	1
	1.1.2 Excitation Contraction Coupling	2
	1.1.3 Ca^{2+} Pools in Smooth Muscle	3
	1.1.4 Extracellular Sources of Ca^{2+}	5
	1.1.5 Intracellular Sources of Ca^{2+}	6
	1.1.6 Relaxation	6
	1.1.7 Ca^{2+} in Heart Tissue	8
	1.2 CALCIUM CHANNEL ANTAGONISTS	10
	1.2.1 Definition and Classification	10
	1.2.2 Therapeutic Use of Calcium Channel Blockers	14
	1.2.3 Mechanism of Action of Calcium Channel Blockers	15
	1.2.4 Sites of Action of Calcium Channel Blockers	18
	1.2.5 SAR of 1,4-Dihydropyridine Calcium Channel Blockers	20
	1.2.6 Calcium Agonists	21
	1.2.7 Selectivity of the Calcium Channel Blockers	24
2.	OBJECTIVE	29
3.	METHODS	32
	3.1 Guinea Pig Ileal Longitudinal Smooth Muscle (GPILSM)	32
	3.2 Isolated Atria Preparations	33
	3.3 Dose Response curves to CD, CA and K^+ in GPILSM	39

3.4 Pharmacological Evaluation of Novel Calcium Channel Blockers 41

3.5 Pharmacological Evaluation of Novel Calcium Agonists 42

3.6 Indirect Blood Pressure Measurement 45

3.7 Materials 45

3.8 Statistical Analysis 46

4. RESULTS AND DISCUSSION 47

4.1 Comparative Effects of Nifedipine on the Isolated Atria of GP, Mouse and Rabbit 47

4.2 Effect of Nifedipine in Conscious Experimental Hypertensive and Normotensive Rats 52

4.3 C4-Pyridinyl Nifedipine Analogs: Pharmacological Evaluation in GPILSM 59

4.4 C4-Pyridinyl Nifedipine Analogs: Pharmacological Evaluation in GP Isolated Atria .68

4.5 C4-Dihydropyridinyl Nifedipine Analogs: Pharmacological evaluation in GPILSM 71

4.6 C4-Dihydropyridinyl Nifedipine Analogs: Pharmacological evaluation in GP isolated atria 83

4.7 C4-Tetrahydropyridinyl Nifedipine Analogs: Pharmacological evaluation in GPILSM 86

4.8 Substitution of the 1,4-Dihydropyridine Ring of Nifedipine by 1,2-Dihydropyridine: Pharmacological Evaluation in GPILSM 91

4.9 C4-Pyridinyl Analogs of Bay K8644: Pharmacological Evaluation in GPILSM and GP Isolated Atria 97

5. CONCLUSION 104

5.1 General Discussion 104

5.2 Future Considerations 109

Bibliography 112

List of Tables

Table		Page
1.1	Classification of calcium channel blockers (Groups I, II, III & IV)	13
3.1	Effect of time and light/dark conditions on the ID_{50} detrermination of nifedipine in GPILSM	41
4.1	Resting heart rate and resting developed tension in GP, mouse and rabbit atria preparations	49
4.2	Comparative effects of nifedipine on the isolated atria of GP, mouse and rabbit	50
4.3	Effect of 4mg/Kg nifedipine i.p. administration on the resting B.P. of WKY and SHR rats	55
4.4	Calcium channel antagonist activity of C4-pyridinyl analogs in GPILSM	60
4.5	Calcium channel antagonist activity of C4-pyridinyl analogs in GP isolated atria	68
4.6	Calcium channel antagonist activity of C4-dihydropyridinyl analogs in GPILSM	71
4.7	Calcium channel antagonist activity of C4-dihydropyridinyl analogs in GP isolated atria	84
4.8	Calcium channel antagonist activity of C4-tetrahydropyridinyl analogs in GPILSM	87
4.9	Calcium channel antagonist activity of 1,2-dihydropyridine analogs in GPILSM	92
4.10	Pharmacological profile of C4-2'-, 3'- and 4'-pyridinyl analogs of Bay K8644	98
5.1	Comparison of GPILSM relaxant activity ID_{50} with binding activity K	108

List of Figures

Figure		Page
1.1	Schematic diagram of cellular mechanisms of Ca^{2+} regulation.	9
1.2	Structural diagrams of compounds designated as Calcium Channel Blockers	11
1.3	Chemical structure of the 1,4-dihydropyridine ring of nifedipine, showing the various positions for changes and substitutions	20
1.4	Chemical structures of the calcium agonists	22
1.5	Structural formulas of some 1,4-dihydropyridine analogs of nifedipine with selective effects	26
3.1	Schematic representation of the GPILSM preparation set up	34
3.2	Tension-response relationships in GP atria	35
3.3	Tension-response relationships in mouse atria	35
3.4	Tension response relationships in rabbit atria	36
3.5	Stimulation rate dependence of left GP atrium	38
3.6	Stimulation rate dependence of left rabbit atrium	38
3.7	Dose response curve of CD	39
3.8	Dose response curve of CA	40
3.9	Dose response curve of K^+	40
3.10	Effect of nifedipine on CD mechanical responses in GPILSM	42
3.11	Dose response curve of nifedipine in GPILSM	44

Figure Page

3.12 Effect of nifedipine on CD dose response curve in GPILSM 44

4.1 % Change in B.P. as a function of time in WKY rats after i.p. administration of nifedipine 53

4.2 % Change in B.P. as a function of time in SHR rats after i.p. administration of nifedipine 54

4.3 Changes in H.R. as a function of time in WKY rats after i.p. administration of nifedipine 56

4.4 Changes in H.R. as a function of time in SHR rats after i.p. administration of nifedipine 57

4.5 Comparison of the 3-dimensional representation of nifedipine and C4-2'-pyridinyl analog 64

4.6 Comparison of the 3-dimensional representation of C4-phenyl and C4-dihydropyridinyl analogs 77

4.7 Recovery of original control responses after inhibition by calcium antagonists 80

4.8 Antihypertensive action of Cpd 32 on conscious SHR rats 81

4.9 Comparison of the 3-dimensional representation of C4-phenyl and C4-tetrahydropyridinyl analogs 89

4.10 Structure and activity of the N-methylated derivative of nifedipine 91

4.11 3-dimensional representation of 1,4- and 1,2-dihydropyridine analogs 94

4.12 Tracing showing reponses to the novel calcium agonists in cardiac and smooth muscle preparations 99

4.13 D-R curve of Cpd 75 with/without the presence of KCl 101

Figure Page

4.14 Extracellular Ca^{2+} dependence of Cpd 75 responses in GPILSM 101

1. LITERATURE SURVEY

1.1 CALCIUM AND MUSCLE CONTRACTION

1.1.1 Regulatory Role of Intracellular Calcium

In the last three decades there has been a steadily increasing awareness of the importance of calcium (Ca^{2+}) as a mediator of many physiological processes. As published in numerous books (Anghileri and Anghileri 1982; Campbell 1983), cytosolic free Ca^{2+} is now known to be one of the messengers responsible for initiating cellular events such as: excitation-contraction coupling of skeletal, smooth and cardiac muscle; action potential and impulse conduction in the myocardium; stimulus-secretion coupling and many metabolic enzyme systems.

Although extracellular Ca^{2+} concentration is in the millimolar range, intracellular free Ca^{2+} is maintained at a low level at rest (10^{-7}M) by various pumps and systems. An increase to about 10^{-5}M initiates the cellular activation which ceases to function when the cytosolic free calcium falls below the critical level.

It is now established that contraction in skeletal muscle can be described by the "Sliding Filament Theory" (Huxley, 1957). This same mechanism is believed to happen in smooth and cardiac muscle. In general, the basic unit of the contractile apparatus consists of thin (actin) and thick (myosin) filaments aligned in parallel with a region of

overlap and cross-bridging between them. During contraction, neither of the two filaments shorten, they however move past each other forming new sites of cross-bridges for tension development. The cross-bridges are the enzymatically active portion of the thick filaments which protrude from it to bind to the thin filaments.

How the increase in cytosolic free Ca^{2+} ultimately leads to the formation of these new cross-bridge sites during contraction has not been fully elucidated yet. However, basically the first step in the intracellular, calcium dependent, regulatory process involves Ca^{2+} binding to the calcium-binding proteins which then activate the enzymes responsible for new cross-bridge formation sites.

Calcium binding proteins that have been isolated are troponin C in striated muscle (Ebashi, 1974), calmodulin (Yagi *et al.*, 1978) and leitonin (Mikawa *et al.*, 1978) in smooth muscle, and calmodulin and troponin C in cardiac muscle.

1.1.2 Excitation Contraction Coupling

Excitation contraction coupling is the process by which excitation of the muscle fibres initiates contraction. In skeletal muscle, release of acetylcholine at the neuromuscular junction depolarises the membrane by increasing its permeability to Na^+ and this sets off an action potential that propagates into the T tubules to the sarcoplasmic reticulum, triggering the release of Ca^{2+} from

the sarcoplasmic reticulum so that contraction ensues.

Smooth muscle can also be activated through depolarization by electical stimuli and through stimulation of pharmacological receptors. Based on what was known from skeletal muscle, electromechanical coupling (i.e. mechanical activity caused by membrane depolarization or action potential, as mimicked by high K^+) was assumed to be the only factor leading to contraction in smooth muscle. However it was later found that contraction in vascular smooth muscle could also be induced with or without small changes of membrane potential by such agonists as acetylcholine and norepinephrine (Evans and Schild, 1957). The term pharmacomechanical coupling was thus coined to describe this phenomenon of contraction with no accompanying depolarization (Somlyo and Somlyo, 1968).

1.1.3 Ca^{2+} Pools in Smooth Muscle

Unlike skeletal muscle where all the Ca^{2+} required for triggering contraction comes from the well developed sarcoplasmic reticulum, in smooth muscle the activating Ca^{2+} can be derived either from intracellular or extracellular sources (Bose and Innes, 1975; Deth and van Breemen, 1977). This concept began with the observation, made by Bohr (1963) that in rabbit aorta, norepinephrine (NE) induced contraction is composed of two phases, an initial fast phase (phasic) and a slower sustained phase (tonic). While the phasic response was augmented in Ca^{2+} free solutions, the

tonic response was proportional to the external Ca^{2+}. Subsequently Hinke *et al.* (1964) noted that high K^+ depolarization contractions were more dependent on extracellular Ca^{2+} than NE induced contractions. Through the use of lanthanum, which displaces extracellularly bound calcium and inhibits transmembrane calcium transport, van Breemen *et al.* (1972) showed that lanthanum abolished the high K^+ contractions, and the tonic but not the phasic component of the NE induced contractions. It was therefore concluded that high K^+ depolarization causes Ca^{2+} influx into the cells through calcium channels and that NE initially releases Ca^{2+} from intracellular stores and then increases permeability to sustain the tonic phase of contraction. Also, since only one phasic NE contraction could be elicited in the presence of lanthanum, it was proposed that the NE mediated Ca^{2+} release was from a limited store which had to be replenished by external calcium. It must however be stressed that the relative dependence of the phasic or tonic component of contraction on extracellular and/or intracellular sources, not only depends on the mode of activation but also on the smooth muscle involved (van Breemen *et al.*, 1980a; Wolowyk *et al.*, 1985), one example being the rat aorta where NE contractions can be repeatedly induced in the absence of extracellular calcium (Heaslip and Rahwan, 1982). Hence it is clear that the terms phasic and tonic should only be used as a descriptive designation of the components of agonist induced

contractions.

1.1.4 Extracellular Sources of Ca^{2+}

Both K^+ and receptor induced contractions involve Ca^{2+} influx into cells. It is believed that two sets of channels through which Ca^{2+} can enter cells exist:

1. Potential operated channels (POC), activated by membrane depolarisation.
2. Receptor operated channels (ROC), activated by agonist receptor occupation with no accompanying depolarization.

The main evidence for these two populations of channels stems from the work of van Breemen *et al*. (1980a) who showed that in rabbit aorta when maximal Ca^{2+} influx (which depends on depolarization) was obtained by high concentration of K^+, addition of NE caused an additional Ca^{2+} influx. Thus, depending on the tissue, an agonist drug may have the ability to activate Ca^{2+} by utilising different relative proportions of these two channels; on the other hand K^+ depolarization may induce release of neurotransmitters from the autonomic nerves present in the smooth muscle (Golenhofen *et al*., 1975) therefore also affecting the ROC. So far there has been no concrete evidence to show whether the ROC or POC are different channels or perhaps just a different conformation of one system (there may also be different subgroups among the ROC and POC themselves).

In addition to Ca^{2+} entry through the ROC and POC, a resting passive Ca^{2+} entry and Na^+/Ca^{2+} exchange mechanisms

also exist (Lang and Blaustein, 1980), however they are not believed to contribute significantly to the contractile event.

1.1.5 Intracellular Sources of Ca^{2+}

Although it is now well established that intracellular Ca^{2+} release by some agonists contribute to contraction, the sources of these intracellular stores are not exactly known. It is however believed that the sarcoplasmic reticulum is the major source because it has been shown that a correlation exists between the volume of sarcoplasmic reticulum in different smooth muscles and their ability to contract in Ca^{2+} free solutions (Basar and Weiss, 1981), and also caffeine (which is known to release Ca^{2+} from the sarcoplasmic reticulum of striated muscle) can release Ca^{2+} in normal and saponin-skinned smooth muscles (Daniel, 1984).

Mitochondria are not believed to contribute to the physiological regulation of cytoplasmic Ca^{2+} because their affinity for Ca^{2+} is relatively low (Somlyo *et al.*, 1974), they do however represent a calcium store of a very high capacity. Another possible source of intracellular Ca^{2+} is membrane bound Ca^{2+} (Sugi and Daimon, 1977), however this is still at a hypothetical stage.

1.1.6 Relaxation

After contraction, relaxation follows when the increased cytosolic free Ca^{2+} is removed. Two different

hypotheses on the mechanism of calcium extrusion have been proposed.

It has been postulated in analogy with the observations made in squid giant axon (Baker, 1972) and on the basis of experimental work on vascular smooth muscle, that Ca^{2+} is extruded from the cytoplasm by a Na^{+}/Ca^{2+} exchange mechanism where the energy is derived from the (inward directed) transmembrane Na^{+} gradient (Blaustein, 1977). The other proposed mechanism involves the Ca^{2+}-ATPase system, as first described in red blood cells (Schatzmann, 1966) where hydrolysis of ATP is used to fuel Ca^{2+} extrusion. In fact Grover *et al.* (1980) have succeeded in showing that purified membranes from various smooth muscles posses a Ca^{2+}-ATPase transport system.

In some smooth muscles, such as the dog coronary artery, guinea pig taenia coli and the rabbit aorta, it was observed that relaxation was not preceeded by any cellular Ca^{2+} loss (van Breemen *et al.*, 1977; Mueller and van Breemen, 1979). Coupled with the fact that Ca^{2+} is sequestered into the sarcoplasmic reticulum during relaxation (Popescu and Diculescu, 1975) and that in rabbit aorta the sequestered calcium cannot be released again by agonists, it is believed that during relaxation calcium is first sequestered into the sarcoplasmic reticulum and subsequently extruded into the extracellular space. However it has also been suggested that in other smooth muscle (rabbit ear artery), a recycling of released Ca^{2+} between

the cytoplasm and the intracellular storage sites exists since repeated contractions in Ca^{2+} free solutions can be elicited in such muscle (Droogmans and Casteels, 1981).

Mitochondrial sequestration of Ca^{2+} during relaxation is thought to be unlikely under normal physiological conditions, but during Ca^{2+} overload (cell damage) mitochondria have been shown to accumulate Ca^{2+} (Somlyo *et al.*, 1979) thus acting as a buffer system.

The various mechanisms of cellular Ca^{2+} regulation are summarised in Fig. 1.1.

1.1.7 Ca^{2+} in Heart Tissue

Heart tissue is controlled by sympathetic and parasympathetic nerve terminals of the autonomic system through secretion of hormones directly to the heart or into the blood. As in other muscles, Ca^{2+} has an important regulatory role in cardiac cellular activities such as spontaneous activity, impulse conduction in the sinoatrial and atrioventricular nodes, and in the contractile process of cardiac cells. In particular the voltage dependent slow calcium influx is believed to be responsible for these events.

The regulation of calcium in cardiac cells has recently been reviewed (Shamoo and Ambudkar, 1984). In summary, during depolarization Ca^{2+} enters through the potential dependent calcium channels which brings about the release of Ca^{2+} from the sarcoplasmic reticulum (Fabiato and Fabiato,

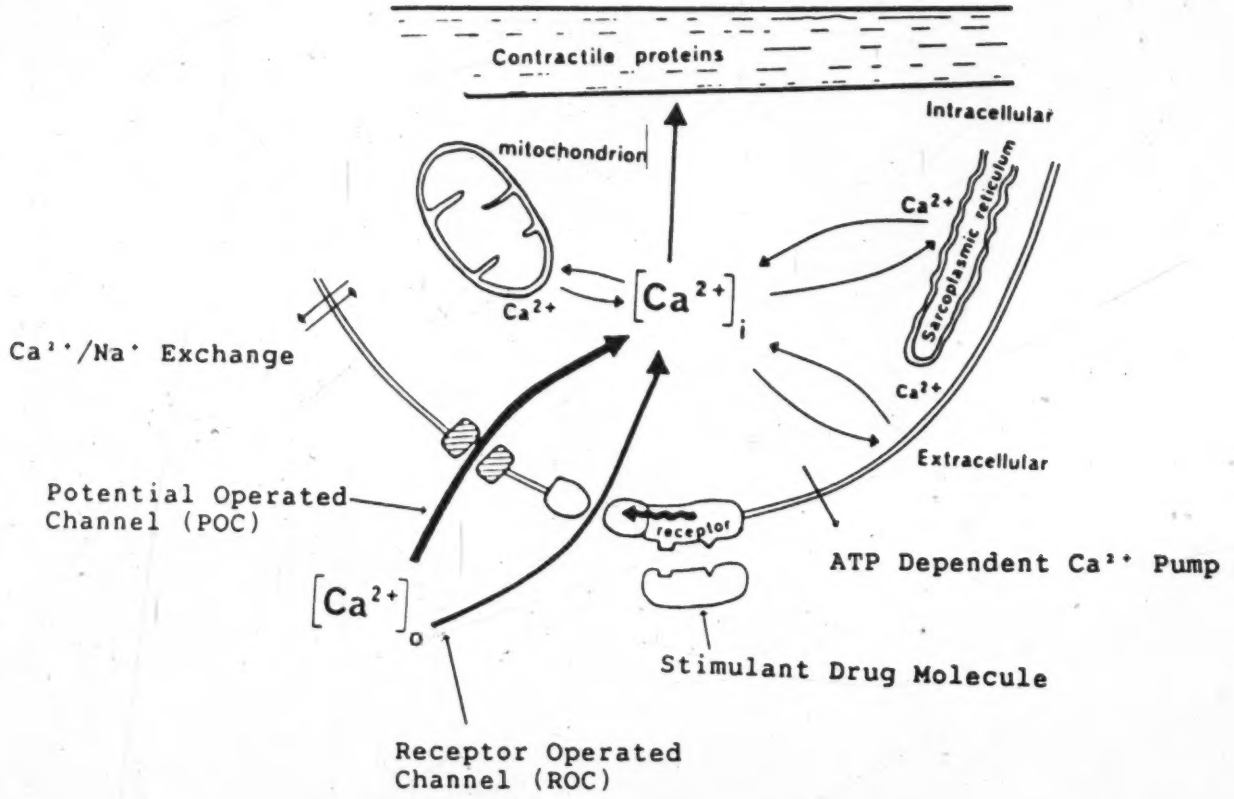


Figure 1.1 Schematic diagram of cellular mechanisms of Ca^{2+} regulation, modified from Casteels, 1980.

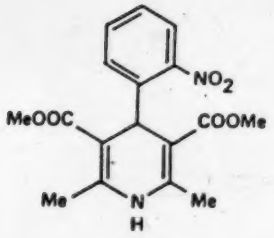
1979). When the cytosolic Ca^{2+} reaches about 10^{-5}M, the troponin inhibition of contraction is removed and contraction ensues. Relaxation follows when the increased cytosolic Ca^{2+} is removed through sequestration into the sarcoplasmic reticulum (Solaro and Briggs, 1974) or extruded from the cell possibly by the sarcolemmal Ca^{2+}-ATPase and Na^{+}/Ca^{2+} exchange systems (Langer, 1982). The mitochondrial Na^{+}/Ca^{2+} exchange system is believed to be only a minor contributor to the regulation of intracellular calcium except during calcium overload.

1.2 CALCIUM CHANNEL ANTAGONISTS

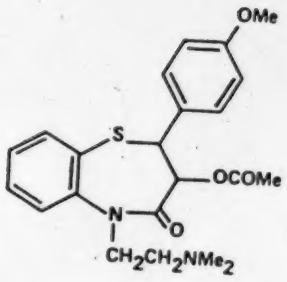
1.2.1 Definition and Classification

In recent years, considerable attention has been directed to a group of compounds known as the Calcium Antagonists. This term was first coined by Fleckenstein (1969) to describe compounds that mimicked the cardiac effects of calcium removal and these effects could be reversed by the addition of Ca^{2+}. Today it is accepted that the main mechanism of action of these compounds is to prevent calcium entry through calcium channels. A variety of structurally dissimilar compounds as exemplified by Diltiazem, Nifedipine and Verapamil in Fig. 1.2 now form part of this group of compounds.

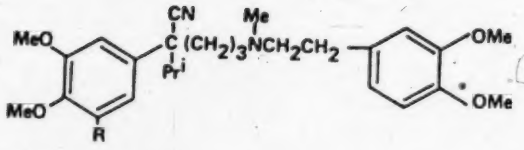
From a pharmacological point of view, the calcium antagonists do not actually inhibit the effects of calcium



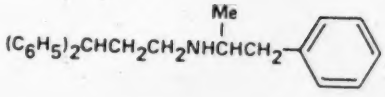
Nifedipine



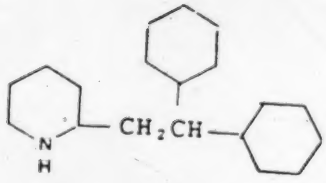
Diltiazem



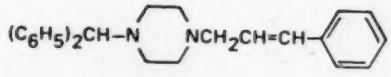
R=H, Verapamil
R=MeO, D600



Prenylamine



Perhexiline



Cinnarizine

Figure 1.2 Structural diagrams of compounds designated as calcium channel blockers.

ions but rather inhibit calcium passage through calcium channels in cell membranes, these compounds are also known as calcium entry blockers (Vanhoutte and Bohr, 1981), slow channel inhibitors and slow channel blockers (Nayler and Poole-Wilson, 1981). Lanthanum (La^{3+}) which binds more readily to Ca^{2+} binding sites than Ca^{2+} itself can be considered as a true calcium antagonist. La^{3+} can inhibit both passive calcium leak and Ca^{2+} entry caused by the calcium ionophore A23187 whereas the calcium channel blockers have no effect on these two modes of calcium entry.

Other than diltiazem, nifedipine and verapamil, numerous compounds such as prenylamine, perhexiline, fendiline, cinnarizine and flunarizine (fig. 1.2) have also been identified as calcium channel blockers. Other compounds like lidocaine and quinidine which effectively block fast sodium channels are also known to weakly impair the slow calcium channels.

Because of the heterogeneity of the calcium channel blockers, attempts have been made to define and classify these compounds (Table 1.1). The original definition as proposed by Fleckenstein in 1969 is now generally accepted, however, according to van Zwieten (1985), the term calcium channel blockers should be limited to those drugs which selectively inhibit the calcium channels such as nifedipine, verapamil and diltiazem. On the other hand, Fleckenstein (1983) suggested that two groups "A" and "B" are required to classify the calcium antagonists; thus group A should

Table 1.1

Classification of calcium channel blockers. (Groups I, II III & IV).

	Nifedipine	Verapamil	Diltiazem	Diphenylalkylamines
van Zwieten	I	I	I	—
Fleckenstein	I	I	I	II
Glossmann	Ia	II	III	Ib
Spedding	I	II	II	III

include the selective calcium channel blockers (nifedipine, verapamil and diltiazem), while group B consist of drugs such as prenylamine and perhexiline which are less potent and less specific (at a concentration that will inhibit 50% of the calcium dependent processes, these drugs will concomittantly impair the fast sodium channels).

Another classification that has been proposed is that of Glossmann *et al*. (1982). Based on ligand binding experiments, four different binding sites are believed to exist and these have been suggested to represent different receptor sites and hence four subgroups of the calcium channel blockers. However Spedding (1985) has proposed that three subclasses possibly exist based on their lipophilicity and functional properties.

It therefore appears at this moment that although it is agreed subgroups of calcium channel blockers exist, no single basis of classifying them is totally satisfactory.

1.2.2 Therapeutic Use of Calcium Channel Blockers

Nifedipine, verapamil and diltiazem have selective cardiovascular effects. Nifedipine is principally indicated for vasospastic angina and hypertension due to its selective vasodilating actions whereas verapamil is used in the treatment of supraventricular tachycardias (Talbert and Bussey, 1983; Frohlich,1985); at a therapeutic dose, nifedipine has less effect on cardiac conduction. Nifedipine has a higher incidence of side effects as opposed to verapamil and diltiazem (overall incidence of side effects reported for nifedipine is 17%, as compared to 9% for verapamil and 4% for diltiazem). Furthermore it is photosensitive and has to be protected from light.

Because some diseases are thought to be due to excessive smooth muscle contraction, which is dependent on calcium, there has been interest in assessing the clinical use of the calcium channel blockers (in particular the 1,4-dihydropyridines) in these conditions. Some of the illnesses for which calcium antagonists might be indicated are cerebral arterial spasm, Raynauds' syndrome, bronchial asthma, esophageal spasm and myometrial hyperactivity (Zelis 1983).

In addition, the potential use of the calcium channel blockers to prevent platelet aggregation has been reported (Brauer *et al.*, 1983). Calcium antagonists have been found to cause reversal of anticancer drug resistance in mice, however the mechanism of this phenomenon is still uncertain (Helson, 1984). They are also being tried clinically in the treatment of mania (Caillard, 1985).

Hence the clinical applications of the calcium channel blockers is potentially vast and with the discovery of new compounds and more clinical studies, one can speculate that the importance of this group of compounds will grow in the future.

1.2.3 Mechanism of Action of Calcium Channel Blockers

Research in the field of calcium channel blockers has been directed in the following areas:

1. Standard Pharmacological Experiments, involving both *in vivo* and *in vitro* methods.
2. Radioligand Binding Studies.
3. ^{45}Ca Flux Studies.
4. Electrophysiological Studies.

Although considerable work has been done, the molecular mechanism of action of these drugs is still unknown, that is how such a diverse group of dissimilar drugs would possess the same end result of preventing calcium influx into the cells. However their high potency, stereoselectivity (Towart *et al.*, 1981a; Echizen *et al.*, 1985) and structure activity

relationships suggest that these compounds bind to specific recognition sites or receptors to mediate their action of channel blockade. The inability of D600 (methoxyverapamil) to exert its action in skinned cardiac and smooth muscles suggests a membrane site of action as opposed to an intracellular action of these compounds (Fleckenstein, 1977).

In general, it is now accepted that all calcium channel blockers preferentially prevent calcium entry through the POC over the ROC. This concept developed from studies which showed that in many vascular tissues such as the rabbit aorta (van Breemen *et al.*, 1980a), rabbit saphenous artery (Towart, 1981b) and the canine coronary artery (van Breemen and Siegel, 1980b), K^+ induced contractions were more sensitive to the calcium antagonists than agonist induced (5-HT, NE and histamine) contractions. Furthermore Farley and Miles (1978) found that in canine trachea, contractions produced by low concentrations of acetylcholine were accompanied by depolarizations and sensitive to verapamil whereas contractions produced by higher concentrations of acetylcholine, without further depolarization, induced a component of the response which was verapamil resistant.

In other smooth muscles such as the guinea pig ileal longitudinal smooth muscle (Rosenberger *et al.*, 1979) and the rabbit basilar artery (Towart, 1981b), both agonist induced and K^+ induced contractions were equally sensitive to the actions of the calcium channel blockers. This suggest

a similarity of action of the K^+ and agonist induced activation in these tissues. Hence the extent of inhibition by calcium channel blockers against agonist induced contractions would be expected to depend on the varying degrees of Ca^{2+} mobilization through POC, ROC and as well as intracellular stores.

However exceptions to this concept of preferential blockade of the POC by the calcium antagonists have been found. Walus *et al.* (1981) have shown that K^+ induced activation of strips of canine mesenteric arteries are less susceptible to nifedipine than are NE contracted strips. Also in the rabbit mesenteric resistance vessels, NE contractions are more sensitive than the K^+ contractures to the inhibitory effect of diltiazem (Cauvin *et al.*, 1982).

Since parallel electrophysiological studies are not available for these isolated smooth muscle experiments, the question of POC selective blockade cannot be fully understood until we know to what extent the agonists induce membrane depolarization in these tissues and also to what degree POC, ROC and intracellular stores are involved.

Other than the principal action of preventing calcium entry into the cells, several "non calcium antagonistic" actions of the calcium channel blockers have been reported. Saida and van Breemen (1983) found that concentrations > 10^{-4}M diltiazem may prevent intracellular Ca^{2+} release. Verapamil and D600 have been reported to inhibit the fast Na^+ current in cardiac and neuroblastoma cells (Bayer *et*

al., 1975). D600 has been shown to inhibit specific receptor ligand binding of alpha adrenergic, muscarinic (Jim *et al.*, 1981) and opiate receptors (Fairhurst *et al.*, 1980).

In general these pharmacological effects are only observed at a much higher concentration of these drugs, and therefore the contribution of these effects to the total pharmacological profile of these drugs is probably insignificant (also when a high concentration of a drug is used, non specific actions can be produced).

1.2.4 Sites of Action of Calcium Channel Blockers

As mentioned earlier, considerable evidence has been accumulated to suggest that the calcium channel blockers bind to specific membrane receptors to produce their pharmacological effects. There is now much interest in isolating and identifying these binding sites by using biochemical techniques. Three peptides (60,000, 54,000 and 30,000 daltons) from chick heart membranes (Rengasamy, 1985) and a 278,000 dalton complex from intestinal smooth muscle (Venter *et al.*, 1983) have been found to copurify with the radio labelled dihydropyridine ligand. If these isolated entities are indeed associated with the pharmacological receptors, then these findings appear to indirectly support the concept that verapamil, diltiazem and nifedipine interact with distinct receptor sites which are allosterically modulated (such allosteric receptors would involve a complex organisation of subunits which are

inter-linked).

The allosteric mechanism of calcium antagonist interaction as proposed by Murphy *et al*. (1983) is based on radioligand binding studies. The radioligand [^{3}H]-nitrendipine, a 1,4-dihydropyridine analog of nifedipine was used in competition experiments to characterize the binding of the 1,4-dihydropyridines to their receptor sites. Verapamil and D600 were found to reduce whereas diltiazem enhanced [^{3}H]-nitrendipine binding; also diltiazem was able to reverse the inhibition caused by D600. Hence it is believed that nifedipine, diltiazem and verapamil bind to distinct separate sites which are linked allosterically to the calcium channel.

Attempts to produce these allosteric actions pharmacologically using *in vitro* isolated tissues have also been made. Recently Yousif and Triggle (1985) found that nifedipine plus diltiazem yielded synergistic antagonism of Ca^{2+} responses in K^+ depolarized guinea pig teania coli and rat mesenteric artery preparations, in parallel to ligand binding experimental results; whereas nifedipine plus D600 produced an additive response. Similarly diltiazem has been shown to potentiate the negative inotropic activity of nimodipine (DePover *et al*., 1983). In contrast, only an additive activity was found between verapamil and nifedipine by Humphrey and Robertson (1983) in the taenia coli and coronary artery preparations; also Spedding (1983) observed additive effects of diltiazem plus nimodipine in taenia

coli.

1.2.5 SAR of 1,4-Dihydropyridine Calcium Channel Blockers

The calcium antagonists for which the structure activity relationships (SAR) have been most studied is the 1,4-dihydropyridine group of compounds, to which nifedipine belongs. Figure 1.3 shows the general structure of this group of compounds. Work in this field has been carried out by Rodenkirchen *et al.* (1978), Loev *et al.* (1974) and Iwanami *et al.* (1979).

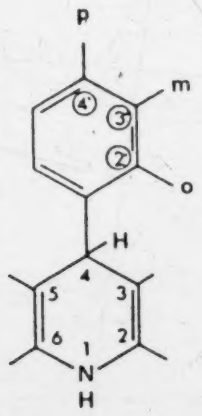


Figure 1.3 Chemical structure of the 1,4-dihydropyridine ring of nifedipine showing the various positions for changes and substitutions.

The general structural requirements for activity can be summarised as follows (Janis and Triggle, 1983):

1. Oxidation of the 1,4-dihydropyridine ring to pyridine abolishes activity.
2. The NH group of the 1,4-dihydropyridine ring must be unsubstituted for optimal activity.
3. The 2,6 substituents of the 1,4-dihydropyridine ring

should be lower alkyl.

4. Replacement of the ester substituents at the 3- and 5-positions by COMe or CN greatly reduces activity.
5. Ester substituents larger than COOMe generally maintain or even increase activity, suggesting a region of bulk tolerance at the site of 1,4-dihydropyridine interaction.
6. The position of the substituent in the C4-phenyl ring is critical, para substitution decreases activity whereas ortho or meta substituents generally increases activity. Activity is generally independent of the electronegativity of the substituent since compounds with electron withdrawing and electron donating groups are active.
7. When the ester substituents at C3 and C5 positions of the dihydropyridine ring are different, the C4 position becomes a chiral centre and stereoselectivity is observed.
8. Perpendicular orientation of the phenyl ring to the 1,4 dihydropyridine ring is probably important for optimum activity.
9. Activity increases with increased ring planarity of the 1,4-dihydropyridine ring.

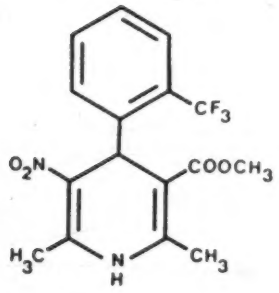
1.2.6 Calcium Agonists

A recent finding by Schramm *et al.* in 1983 has added tremendous excitement to the study of the dihydropyridine compounds. One novel dihydropyridine analog, Bay K8644 (Fig.

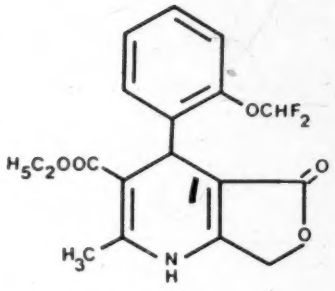
1.4) was observed to produce positive inotropic action and vasoconstrictor effects, that are totally opposite to those of nifedipine. Due to the structural similarity of nifedipine and Bay K8644, it was believed that they both bind to the same sites to mediate their actions.

In fact, considerable evidence now exists to suggest that Bay K8644 produces its effects by promoting calcium influx into cells whereas nifedipine and analogs prevent it. Thus Bay K8644 is known as a "calcium agonist". It has been shown to be pharmacologically dependent on extracellular Ca^{2+}, to antagonise competitively the action of nifedipine and non competitively against verapamil and diltiazem (Su *et al.*, 1984). In ligand binding experiments, Bay K8644 inhibited [^{3}H]nitrendipine binding in a competitive fashion, and its effective concentration in binding studies was found to correlate with its inotropic action in the canine cardiac muscle (Vaghy *et al.*, 1984). It does not seem to have any intracellular calcium release action (Thomas *et al.*,1985), and is believed to promote calcium influx through the POC (Yamamoto *et al.*, 1984; Sanquinetti and Kass, 1984). Through electrophysiological studies, Hess *et al.* (1984) suggested that Bay K8644 promotes a mode of gating where the channels exhibit very long openings while with nitrendipine, the channels are unavailable for opening.

The term "calcium agonist" was coined by Schramm *et al.* (1983) to describe Bay K8644 which produced positive inotropic, positive chronotropic and vasoconstrictor



Bay K 8644



CGP 28392

Figure 1.4 Chemical structures of the calcium agonists.

effects. In fact prior to Schramm' s report, a 1,4-dihydropyridine compound, YC-170 (2-[2-pyridyl]ethyl 4-[o-chlorophenyl]-2,6-dimethyl-5-phenylcarbamoyl-1,4 dihydropyridine-3-carboxylate) was reported to have vasopressor effects in anaesthesized rats and dogs as well as vasoconstrictor effects in isolated rabbit aorta (Takenaka and Maeno, 1982), and therefore YC-170 can probably be considered as a calcium agonist.

Another compound that is known to be a calcium agonist is CGP 28392 (Truog, 1983) as shown in Fig. 1.4. CGP 28392 is, however less potent than Bay K8644.

Recently Lang and Triggle (1985) studied the crystal structures of the calcium agonists and antagonists using X-ray diffraction analysis. They showed that common conformational features (such as boat shaped conformation of the dihydropyridine ring and rotational restriction of the aryl ring), present in calcium channel blockers were also

present in the calcium agonists, suggesting that these features were common for the drugs to bind to their receptors. However, what is observed in the crystal state of these compounds might not reflect their conformational state when they interact with their relevant receptors. Furthermore Lang and Triggle believed that the 3- and 5-substituents of the 1,4 dihydropyridine ring might dictate the agonist or antagonist property of the drug since the (-) and (+) enantiomers of a novel dihydropyridine compound (isopropyl 4-(2,1,3,benzoxadiazol-4-yl)-1,4-dihydro 2,6-dimethyl-5-nitro-3-pyridine caboxylate) exhibited antagonist and agonist properties respectively (Hof *et al.*, 1985).

The fact that we now have a series of agonist and antagonist 1,4-dihydropyridines which seem to bind to the same sites, supports the concept that such 1,4-dihydropyridine receptors exist. If so, some endogenous ligands which bind to these receptors may exist as well, however evidence for their existence is still to be found.

1.2.7 Selectivity of the Calcium Channel Blockers

An intriguing property of the calcium channel blockers is their tissue and organ specificity, and therefore considerable attention has been given to this aspect (Cauvin *et al.*, 1984; Triggle and Janis, 1984; Hof, 1984).

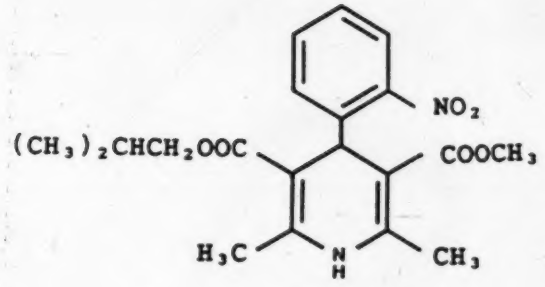
Verapamil is principally indicated for arrhythmias due to its selective action on the cardiac conducting system (frequency dependent), while nifedipine is used in angina

and hypertension because of its specific vasodilating effects. More interestingly, specificity is observed among the 1,4-dihydropyridine group of compounds. Figure 1.5 displays some 1,4-dihydropyridine analogs of nifedipine which have been reported to posses selective effects. Thus niludipine is reported to show enhanced smooth muscle selectivity relative to nifedipine (Hashimoto, 1979), and nisoldipine is also more potent than nifedipine as a vasodilator but has the same cardiac activity (Kazda, 1980). In addition specificity appears to exist among other smooth muscles or vascular beds since nimodipine has been shown to be more selective for the cerebral vasculature (Towart, 1981b), and nisoldipine is more potent in rabbit portal vein than in aorta (Kazda, 1980).

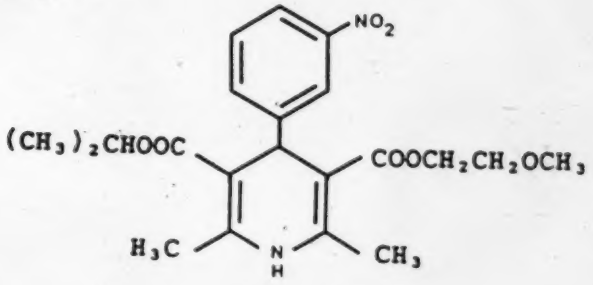
In the rat aorta, contraction produced through α_2 receptors (clonidine) and K^+ are completely sensitive to D600 while α_1 receptor responses are not (Nghiem *et al.*, 1982a). In canine trachea, contractions to low concentrations of acetylcholine are sensitive whereas high concentration responses are insensitive to the action of calcium antagonists (Farley and Miles, 1978).

From what is known today, it appears that specificity is a property of tissue, stimulus or the calcium antagonist and therefore the end result could be a mixture of these three factors.

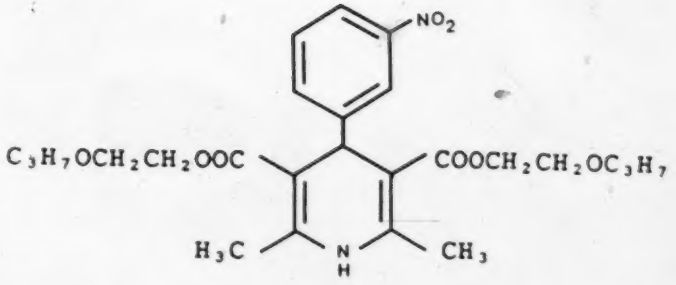
As mentioned earlier, the main mechanism of action of calcium channel blockers is to prevent calcium influx



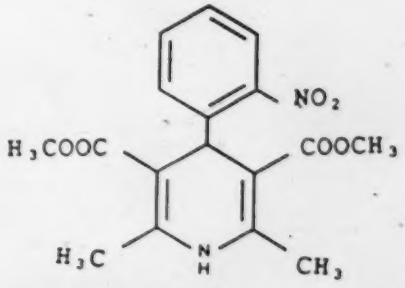
Nisoldipine



Nimodipine



Niludipine



Nifedipine

Figure 1.5 Structural formulas of some 1,4-dihydropyridine analogs of nifedipine with selective effects.

through the POC, hence it is clear that tissues whose activation are more POC dependent would be more susceptible to the action of calcium channel blockers , while tissues that depend on ROC or intracellular Ca^{2+} would be resistant. On the other hand the extent of the inhibitory effect of a calcium antagonist in a particular tissue will depend on the type of stimulus, that is whether the stimulus will activate POC, ROC or intracellular Ca^{2+} release.

Another basis for selectivity could be the existence of different subgroups or subtypes of POC and/or ROC channels with different susceptibility to the calcium channel blockers. Thus the ROC in different tissues are believed to vary in sensitivity while the POC are generally equally sensitive to the action of the calcium channel blockers (Cauvin *et al.*, 1983), except that many K^+ depolarization events in stimulus-secretion coupling are resistant to the calcium antagonists (Triggle and Swamy, 1983).

The fact that many individual calcium channel blockers also have an additional "non Ca^{2+} blockade" action, might contribute to the selectivity observed. For example, nimodipine and nitrendipine have been reported to stimulate the Na^+,K^+ ATPase of smooth muscle membranes whereas nifedipine was without effect (Pan and Janis, 1984).

Specificity *in-vivo* may also depend on factors such as pharmacokinetic differences, physico-chemical properties of the drug, physiological reflexes (e.g. increased heart rate in response to decreased arterial blood pressure) and

differences in innervation of the specific organ.

As reflected in this literature survey, considerable progress has been made in the field of calcium channel blockers in the last decade, and it is anticipated that with more work, it should be possible to understand the regulation of the calcium channels and it may also be possible to design specific calcium channel blockers for specific diseases.

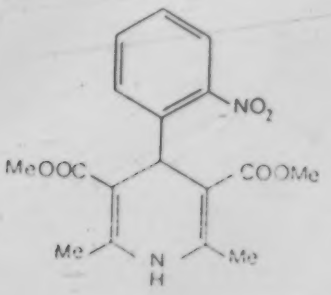
2. OBJECTIVE

The main objective of this project concerns the pharmacological evaluation of novel reduced pyridines that are structurally related to nifedipine, in an attempt to further investigate the structure activity relationships of this group of calcium channel blockers.

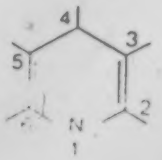
In addition to SAR results, structural manipulation of the parent compound nifedipine could lead to a better drug with desirable properties such as improved chemical stability, less side effects, high potency and more selectivity for specific disorders. SAR studies may also provide a better understanding of the receptor binding sites and the calcium channel itself.

The novel compounds to be evaluated, were synthesized by Dr. Knaus' medicinal chemistry group, composed of Dr. E. Knaus, L. Dagnino, D. Soboleski and Dr. H. Wynn.

The structural changes investigated included:



NIFEDIPINE



1,4 DIHYDROPYRIDINE

1. Replacement of the C4-ortho nitrophenyl ring of

nifedipine by other ring systems: 2'-, 3'-, 4'-pyridinyl, and 3'-, 4'-(1'-substituted-dihydropyridinyl), and 5'-, 2'-, 4'-(1',2',3',6'-tetrahydropyridinyl).

2. Substitution of the methoxycarbonyl groups at either or both C3 and C5 positions by other alkoxy ester substituents, CN and N,N-dimethylaminoethoxycarbonyl.
3. 1,2-dihydropyridine ring in place of the 1,4-dihydropyridine ring of nifedipine.
4. Furthermore the effect of replacing the C4-ortho-trifluoromethylphenyl ring of the calcium agonist Bay K8644 by 2'-, 3'- and 4'-pyridinyl will be determined.

All novel compounds would be evaluated on the GPILSM. The GPILSM preparation was chosen as the principal test system because it has previously been shown that in this preparation, the muscarinic agonist and the KCl-depolarization induced contractions are dependent on extracellular calcium entry which are very sensitive to nifedipine and related analogs (Rosenberger *et al.*, 1979). Also the pharmacological actions of these analogs in the GPILSM are believed to be mediated through a common binding or receptor site (Bolger *et al.*, 1983). Furthermore, the fact that six to eight GPILSM preparations could be used from each animal and since the time course of response as well as the dose cycle used was short compared to other isolated tissues, made this preparation most efficient.

In the GPILSM, the dose of the novel calcium channel blockers that inhibits 50% of the maximal muscarinic and/or KCl induced calcium dependent tonic contractions will be determined, (i.e. their ID_{50}). For the novel calcium agonists, the dose that produces 50% of the maximal response will be obtained, (i.e. their ED_{50}).

Some novel calcium channel blockers will also be evaluated for their cardiac effects on the isolated atria preparations of the guinea pig. This will be carried out to see whether a differential SAR exists between the cardiac effect and the GPILSM relaxant effect. Such studies could lead to the design and synthesis of a drug with selective vasodilating action and higher therapeutic index. Also information obtained from the negative chronotropic SAR may be useful for the design of antiarrhythmic agents.

Novel calcium agonists will also be evaluated on cardiac preparations. SAR for the positive inotropic activity of the calcium agonists could be useful in designing drugs which would improve cardiac contractility and efficiency in conditions such as congestive heart failure.

3. METHODS

3.1 Guinea Pig Ileal Longitudinal Smooth Muscle (GPILSM)

Male guinea pigs (Charles River, Quebec) weighing between 400-600g were killed by a blow on the head and then decapitated. The abdomen was opened longitudinally and the caecum was lifted and placed on the left hand side of the animal. The ileum was cut at the ileo-caecal junction and 20 cm of ileum was removed and placed in Hepes buffered physiological saline solution (HPSS) at 37°C. The HPSS had the following composition (mM/L): NaCl 137.0, $CaCl_2$ 2.6, KCl 5.9, $MgCl_2$ 1.2, Glucose 11.9, Hepes-NaOH (pH 7.4) 9.0.

The longitudinal smooth muscle which is the outer smooth muscle layer of the ileum was separated by a similar method previously described by Rang (1964). A 15 cm length of the ileum was drawn and gently stretched over a glass rod and the mesentery was removed if necessary. Using a blunt scalpel two incisions were made; one on each side of and parallel to the line of the mesentery attachment. The blunt incisions were made only through the longitudinal muscle layer down to the circular one. With a cotton swab, the ileum was brushed tangentially away from the mesentery attachment from one incision line to the other. The longitudinal muscle could be seen to detach from the underlying layer. It was then lifted at one end and by gentle tension was isolated as an intact sheet. Care was exercised not to stretch the muscle at any stage as this could result in a

poor preparation.

Two cm muscle portions, tied at both ends by threads were suspended and fixed at the bottom end in jacketed glass organ baths of 10 ml capacity containing oxygenated HPSS (aerated by 100% O_2) at 37°C as shown in Fig. 3.1. The temperature was maintained by a constant temperature circulator (Haake Model E52). The upper end of the muscle was connected to a Force transducer (Grass FT03) under a resting tension of approximately 300 mg. Isometric tension was then recorded on a Grass polygraph (Model 7D). After an equilibration period of about 45 mins during which the physiological medium was changed every 15 mins, the desired experiments were carried out.

3.2 Isolated Atria Preparations

A guinea pig (GP), mouse (DBF1) or rabbit (New Zealand White 1.5-2.0 Kg) was sacrificed by the method described previously. The chest wall was cut open and the heart removed and placed as quickly as possible in HPSS at 37°C. The atria were carefully dissected and suspended in a 10 ml capacity organ bath to record isometric tension.

In other experiments the two atria were separated and suspended in separate organ baths. The starting length/tension relationship for the atrial preparations were determined and are shown in Fig. 3.2, 3.3 and 3.4. These experiments were carried out by varying the preset tension and measuring the developed force. The tension that was used

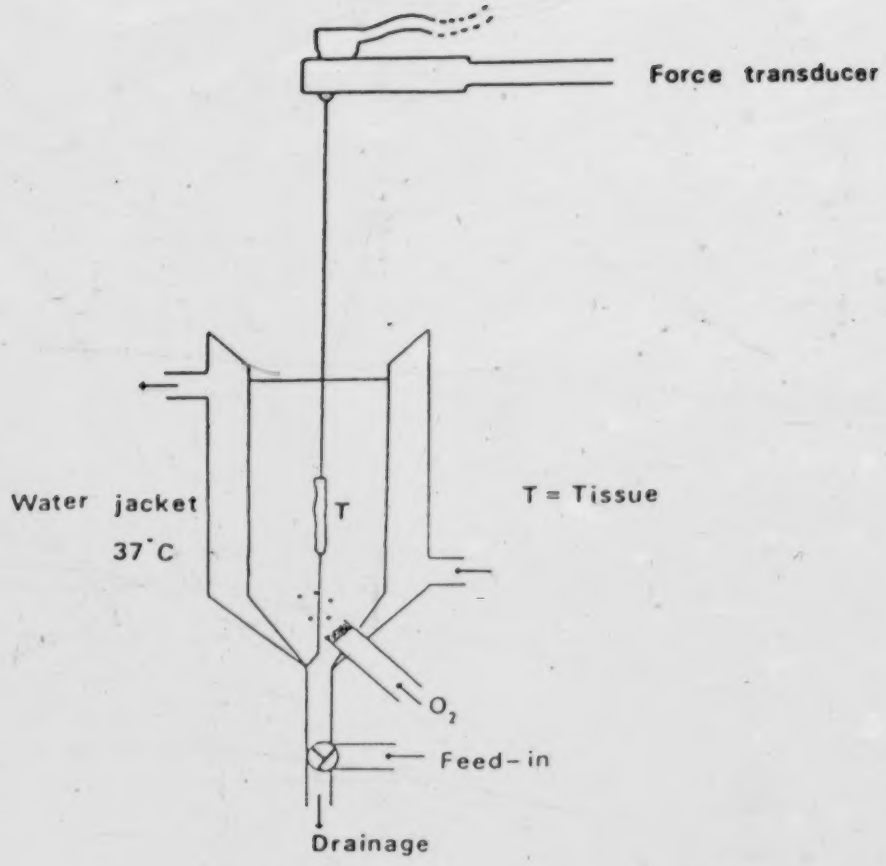


Figure 3.1 Schematic representation of the GPILSM preparation set up.

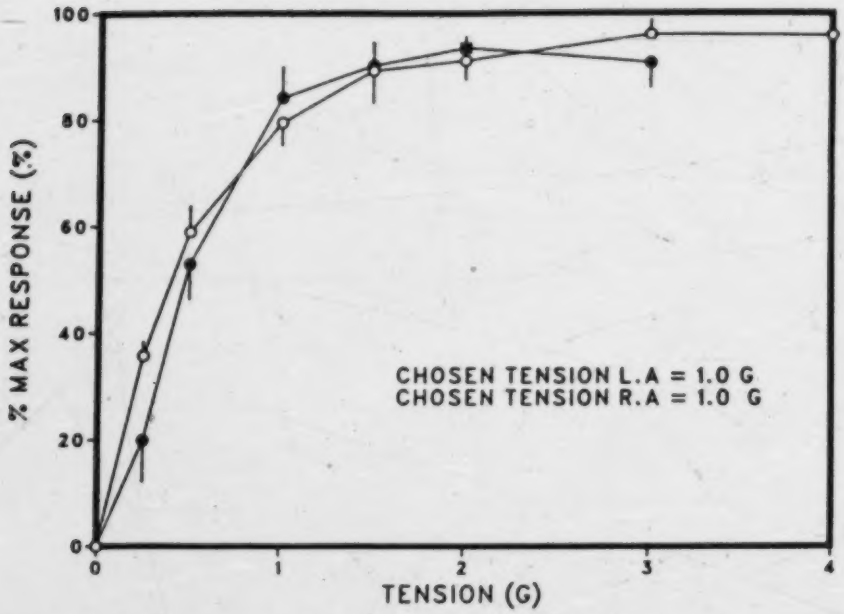


Figure 3.2 Tension response relationships in the guinea pig (•) right and (o) left atria. Each point represents the mean of 5 experiments ± SEM.

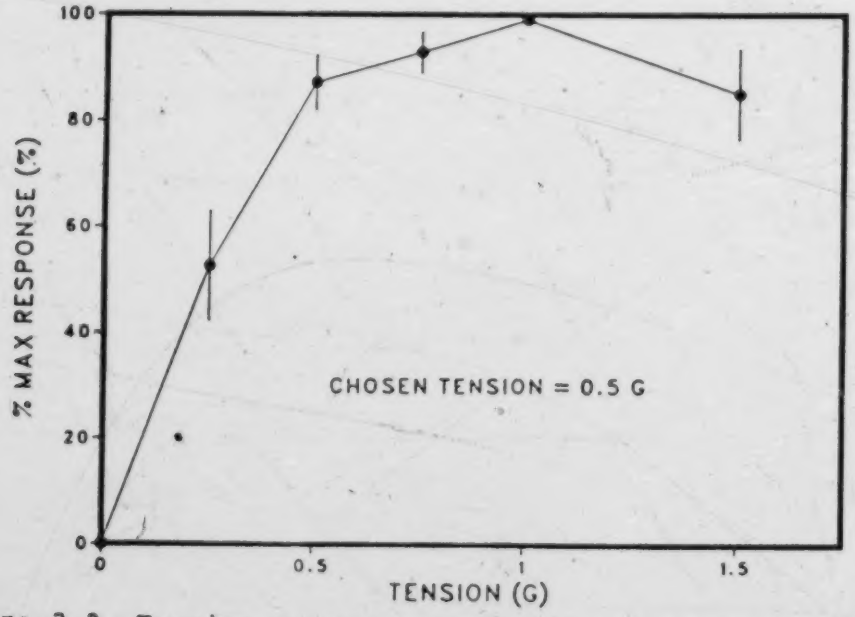


Figure 3.3 Tension response relationships in the mouse atria. Each point represents the mean of 8 experiments ± SEM.

for the subsequent experiments was chosen such that it corresponds to about 85-95 per cent of the maximum effect in the Tension Response curve. Thus the chosen tensions were 1.0 G for the GP atria, 0.5 G for the mouse atria and 3.0 G for the rabbit atria.

The isolated left atrium was electrically stimulated at a constant rate by square pulses of 1.5 msec duration and an intensity approximately twice threshold (threshold = 2.0 volts). The pulses were delivered by an electronic stimulator (Grass S44) through an isolation unit (Grass SIU5) at a stimulation rate of 2 Hz; this value was derived from experiments where the effect of stimulation rate on the inotropic activity of the left isolated atrium was determined. Figure 3.5 and Fig 3.6 illustrate the results of such experiments in the GP and rabbit respectively; 2 Hz corresponds to about 85-95 per cent of the maximal activity.

After an equilibration period of about 45 to 60 mins, the experimental procedure was started. The test drug (agonist or antagonist) was added cummulatively and the inotropic effect measured. The spontaneously beating right atrium or the paired atria were used in a similar fashion to record both inotropic and chronotropic effects.

Results were calculated as % increase or decrease of the starting inotropic contractility or chronotropic rate of the atria.

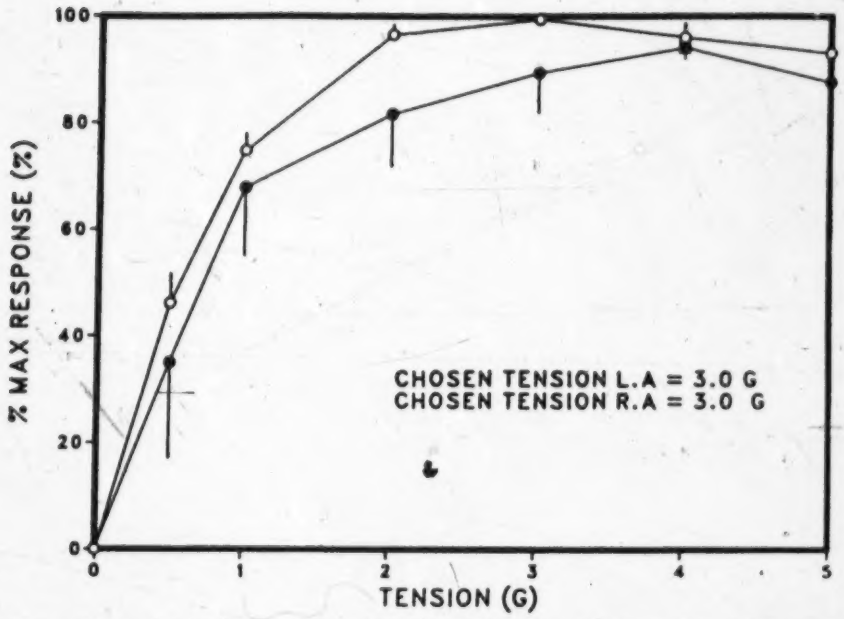


Figure 3.4 Tension response relationships in the rabbit (o) right and (•) left atria. Each point represents the mean of 6 experiments ± SEM.

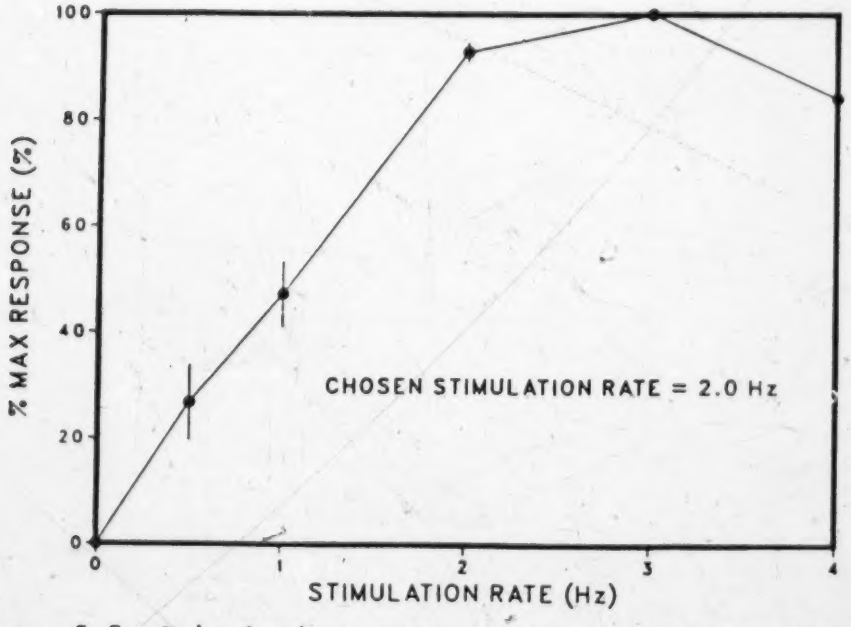


Figure 3.5 Stimulation rate dependence of the left guinea pig atrium. Each point represents the mean value ± SEM, of 6 experiments.

3.3 Dose Response curves to CD, CA and K^+ in GPILSM

Cummulative dose response curves were obtained to the muscarinic agonists cis-2-methyl-dimethylaminomethyl-1,-3-dioxolane methiodide (CD), carbachol (CA), and high K^+ in GPILSM, using the method described by van Rossum (1963). Doses of the agonists were added in a geometrical fashion in steps of one half log 10. The next dose was not added until the response to the previous dose had reached a plateau. The total volume of solution added to the tissue bath during such an experiment did not exceed more than 4% of the total bath volume.

From the dose response curves obtained (Fig. 3.7, 3.8 and 3.9) the maximal dose was determined for each agonist. Thus the maximal doses were $5X10^{-7}$M for CD, $5X10^{-7}$M for CA and 80 mM for KCl. These maximal doses were chosen as the control doses for subsequent experiments where the ID_{50} of test drugs (antagonists) were evaluated (ID_{50} is defined as the concentration of the antagonist that will inhibit the control response by 50%).

Since it is believed that calcium channel blockers are less active in hyperosmolar solution (Hof and Vuorela, 1983), isotonic 80 mM KCl HPSS was prepared by substituting NaCl by KCl. The 80 mM KCl physiological solution had the following composition (mM/L): NaCl 65.0, $CaCl_2$ 2.6, KCl 80.0, $MgCl_2$ 1.2, Glucose 11.9, Hepes-NaOH (pH 7.4) 9.0.

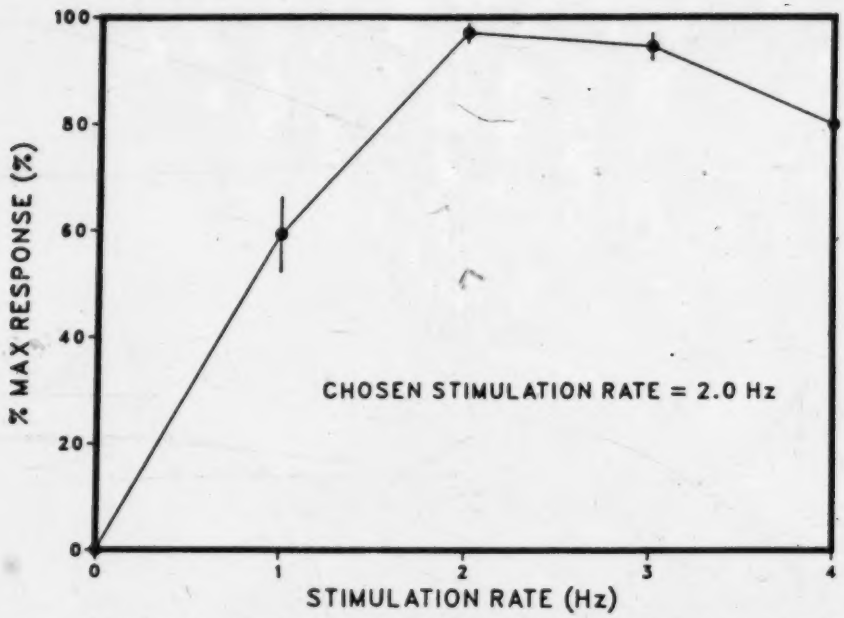


FIgure 3.6 Stimulation rate dependence of the left rabbit atrium. Each point represents the mean value ± SEM, of 6 experiments.

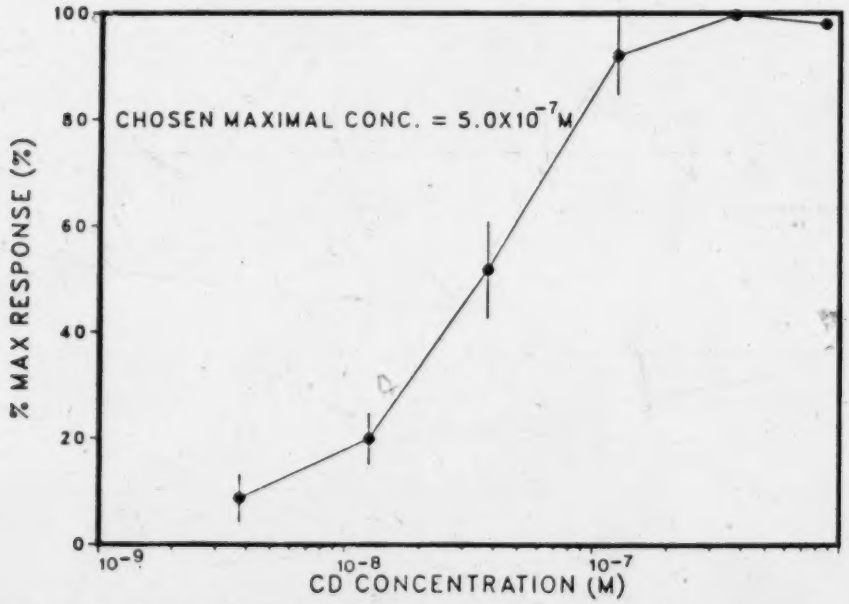


Figure 3.7 Dose response curve of CD. Each response represents the mean value ± SEM, of 6 experiments.(GPILSM).

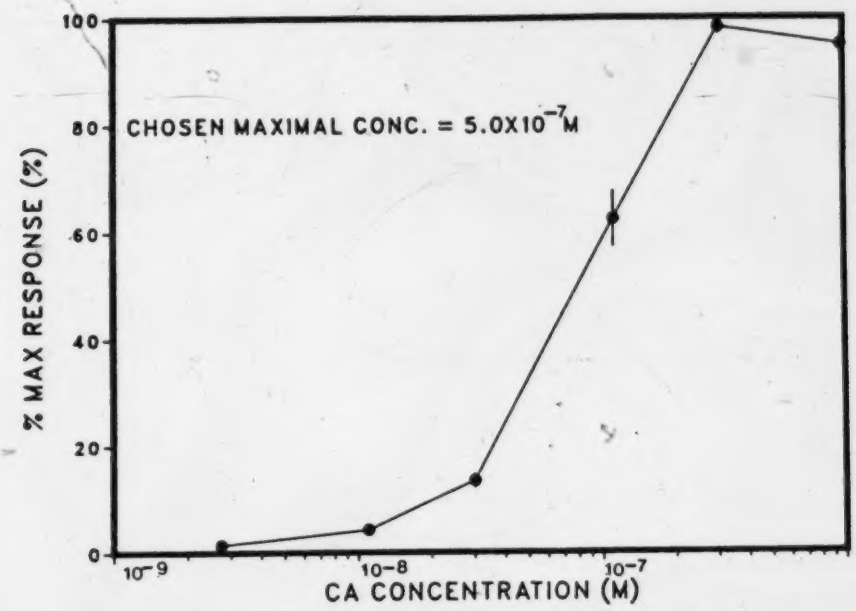


Figure 3.8 Dose response curve of CA. Each point represents the mean value ± SEM, of 8 experiments.(GPILSM).

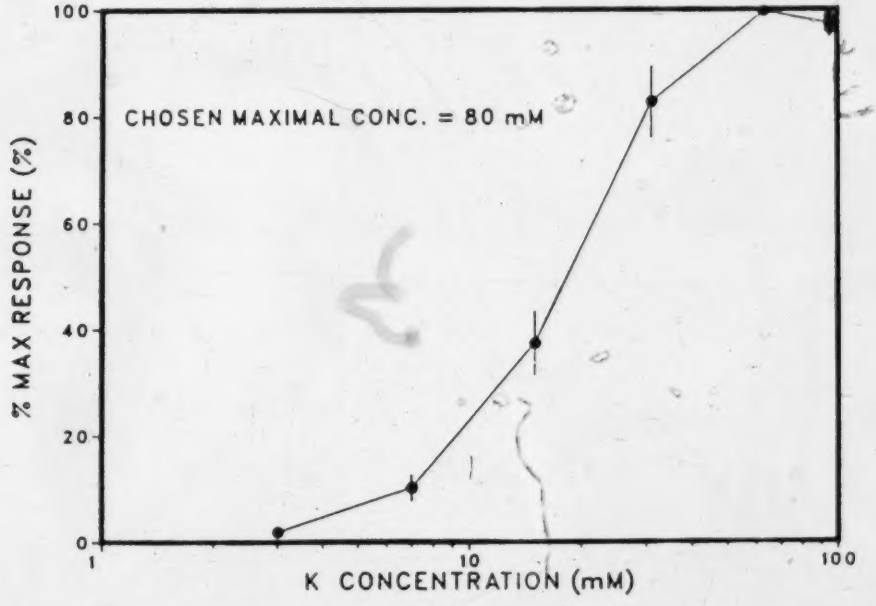


Figure 3.9 Dose response curve of K^+. Each point represents the mean value ± SEM, of 5 experiments.(GPILSM).

3.4 Pharmacological Evaluation of Novel Calcium Channel Blockers

All novel calcium channel blockers were assesed on the GPILSM, in addition some test drugs were also evaluated on the GP isolated atria.

Novel calcium channel blockers were evaluated on the GPILSM by determining their ID_{50} using the following procedure. Constant maximal responses were obtained to the control agonist at 15 mins intervals. The average of at least two tonic control responses was taken as the 100% response. The test drug (i.e antagonist) was then added to the tissue bath and incubated for 10 min before the control dose was repeated. This part of the experiment was carried out in a darkened room (see following paragraph). The % inhibition of the maximal response was then calculated and the ID_{50} was derived graphically by linear regression analysis of 3 points on the linear portion (20 to 80% inhibition) of the dose response curve of the antagonist.

Nifedipine is known to undergo spontaneous and photo-degradation (Antman *et al.*, 1983). Since no report on the effect of light on the ID_{50} determination of nifedipine, has been reported, experiments were undertaken to compare the effects of various incubation conditions (10 min in light, 10 min and 30 min in darkened room) on the ID_{50} determination of nifedipine. The results are displayed in Table 3.1. No statistically significant differences were found between the values obtained from the three methods of

incubation. However to avoid the possibility of photo-degradation, a 10 min dark incubation condition was adopted in this study.

As exemplified by nifedipine, Figs. 3.10 and 3.11 show the effect of a calcium channel blocker on the mechanical response and dose response curve respectively of an agonist (CD). The ID_{50} of nifedipine was derived graphically from its dose response curve shown in Fig. 3.12.

A typical response to CD as depicted in Fig. 3.10 consists of what is described as the phasic and tonic response. As the tonic response is associated with extra-cellular calcium entry, only this component was considered in this study. Blockade of the tonic response is observed in the presence of nifedipine, and after washing out, maximal doses of the control agonist were repeated every 15 min until the original contractions were regained. The time taken to achieve recovery was noted.

Some test drugs were also added cummulatively to atrial preparations to determine their negative inotropic and chronotropic effects. ID_{50} or ID_{25} were calculated graphically by linear regression analysis.

3.5 Pharmacological Evaluation of Novel Calcium Agonists

Novel calcium agonists were assesed on the GPILSM and the GP atrial preparations. Cummulative doses of the test drugs were added as mentioned earlier, to a resting GPILSM or to an atrial preparation. ED_{50} for the positive inotropic

Table 3.1

Effect of 3 different incubation protocols: 10 min light, 10 min dark and 30 min dark on the ID_{50} determinations of nifedipine. No statistical significance was obtained between the ID_{50} values using the 3 protocols at the 5% probability level.(GPILSM).

	INCUBATION PROTOCOLS		
	10 MIN LIGHT	10 MIN DARK	30 MIN DARK
Mean (ID_{50})	$1.19X10^{-9}M$	$1.16X10^{-9}M$	$8.61X10^{-9}M$
SEM	± 0.25	± 0.22	± 1.60
No. of Exp.	5	5	5

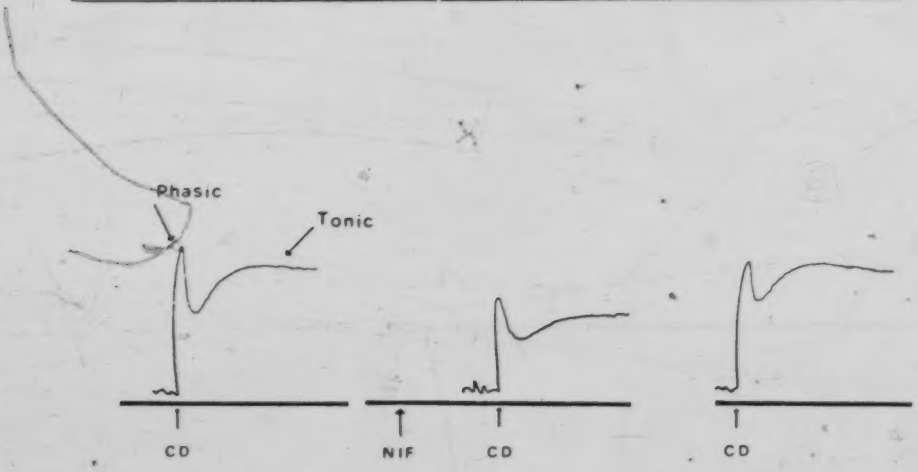


Figure 3.10 Effect of nifedipine on the CD mechanical responses in GPILSM.

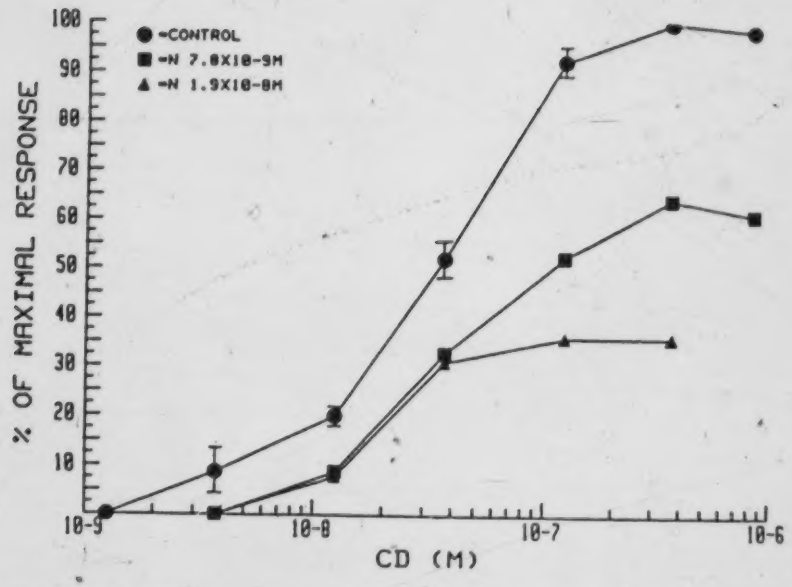


Figure 3.11 Effect of nifedipine on CD dose response curve, showing inhibition of the CD maximal response in presence of nifedipine.(GPILSM).

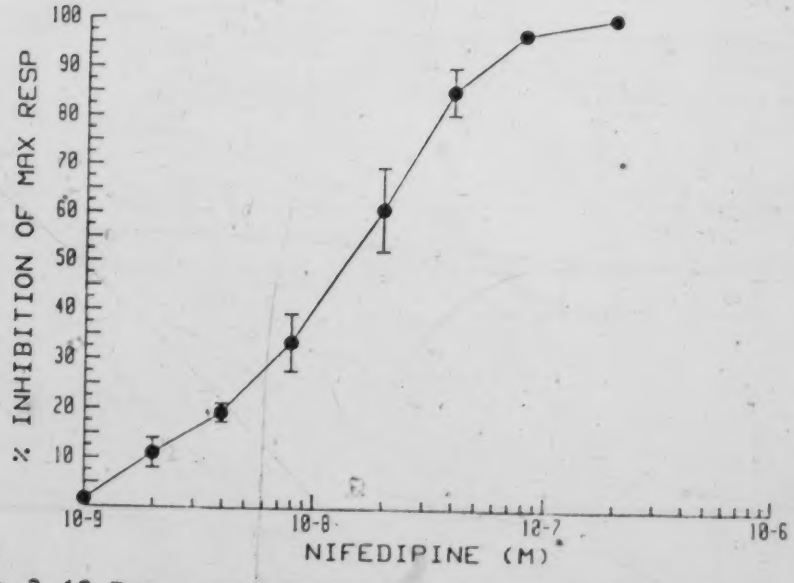


Figure 3.12 Dose response curve of nifedipine.(GPILSM).

and positive chronotropic effect, and the ED_{50} for the contractile activity in the GPILSM were then obtained.

3.6 Indirect Blood Pressure Measurement

Spontaneously hypertensive male rats (SHR) developed by Okamato and Aoki (1963) from Japanese Wistar rats and the corresponding Kyoto normotensive controls (WKY) were supplied by Charles River. They were then bred and maintained at the Animal Care Unit, University of Alberta.

A conscious rat (> 14 weeks) was restrained in a cage and prewarmed on a heated plate (32°-35°C) and with an overhead lamp. The rat was kept this way during the whole experiment. Systolic blood pressure was indirectly measured by the tail-cuff method employing a pulse transducer and an electrosphygmomanometer (Grass Model 7P8H) on a Grass polygraph. Each blood pressure determination was the mean of at least three recordings. B.P measurements were made predose and after the B.P had stabilised. After i.p injection of nifedipine or the test drug the B.P was followed for the next three hours. Results were calculated as % decrease or increase of the predose systolic B.P.

3.7 Materials

All chemicals were purchased from Sigma, except CD (a gift from Dr. C. Triggle, Memorial University of Newfoundland, St. John's) and the novel dihydropyridines including nifedipine were synthesized at the Faculty of

Pharmacy, University of Alberta by Dr. Knaus' medicinal group. The novel compounds were first dissolved in DMSO or 95% ethanol to produce 10^{-2}M solutions and thereafter diluted in distilled water if possible. At concentrations used in this study, DMSO and ethanol were shown not to affect the control responses. Test solutions were freshly prepared prior to the experiments and the containers were wrapped with tin foil to protect the test solutions from light.

3.8 Statistical Analysis

Results are reported as arithmetic means (± SEM, unless otherwise indicated). Comparisons of mean values were made by use of the student' s unpaired t-test. A statistically significant difference was considered to exist for p values less than 0.05.

4. RESULTS AND DISCUSSION

4.1 Comparative Effects of Nifedipine on the Isolated Atria of GP, Mouse and Rabbit

Since one of the goals of this project was to evaluate the cardiac effects of novel calcium channel blockers, experiments were first carried out to compare the cardiac effects of the classical calcium antagonist nifedipine on the isolated atria of the GP, mouse and rabbit, in an attempt to find a most economical and sensitive test preparation.

Purely from a cost point of view GP were most expensive and mice were the cheapest. The cost of purchase of the animals was $20 for each GP, $12 for each rabbit and $1.50 for each mouse; while the maintenance cost for the animals per day was 32 cents for each GP, 65 cents for each rabbit and 17 cents for a cage of 5 mice. However, the relative effectiveness of these preparations had to be considered before choosing the least costly.

Isolated atrial preparations were used as the test model because they allowed both inotropic and chronotropic effects to be recorded. In the rabbit, the right and left atria were separated to record chronotropic and inotropic actions respectively. However the small size of the mouse atria only permited the use of paired atria in monitoring both inotropic and chronotropic effects together. With respect to the guinea pig, both the paired and unpaired

atria preparations could be used. As a result, the GP cardiac preparations served as the comparative control between the three species. (i.e, comparison of the mouse paired atria vs. the GP paired atria and the rabbit unpaired atria vs. the GP unpaired atria).

Table 4.1 lists the resting heart rate and the resting developed tension, obtained for the different test systems investigated. Thus the isolated paired atria of the mouse was observed to have the fastest resting heart rate of 291 beats per min, in contrast this preparation also had the lowest resting developed tension of 0.028 G. The rabbit atria preparation was found to posses a resting heart rate of 110 beats per min and a resting developed tension of 0.30 G, both these values were lower than those obtained for the GP atria preparations.

Table 4.2 displays the negative inotropic and negative chronotropic actions of nifedipine in the isolated atria preparations of G.P, mouse and rabbit. It is interesting to note that in the guinea pig the negative inotropic effect of nifedipine was more pronounced in the isolated unpaired atria than the paired atria.

Since it was established in section 3.2 that inotropic activity depends on stimulation frequency, the electrically driven left atrium was used to assess the negative inotropic effect in all subsequent experiments. This was done to avoid the complication of interpretating altered inotropic responses with concomitant changes in chronotropic activity.

Table 4.1

Resting heart rate and resting developed tension in the various atria preparations. Data are given as mean ± SEM. Number in parentheses represents number of experiments.

System	Developed Tension G	Heart Rate Beats/mim
Guinea pig Paired atria	0.44 ± 0.07 (4)	176 ± 11 (4)
Mouse Paired atria	0.028 ± 0.003 (8)	291 ± 20 (8)
Guinea Pig Unpaired atria	0.41 ± 0.04 (6)	134 ± 6 (5)
Rabbit Unpaired atria	0.30 ± 0.03 (4)	110 ± 10 (4)

Table 4.2

Comparative effect of nifedipine on the isolated atria of GP, mouse and rabbit. ID_{25}- concentration required to induce 25% inhibition of the contractile activity or heart rate. ID_{50}- concentration required to induce 50% inhibition of the contractile activity in the left isolated atria. Data are given as mean ± SEM. Number in parentheses represents number of experiments.

System	Inotropic Effect (C.F)	Chronotropic Effect (H.R)
Guinea pig Paired atria	ID_{25}= $9.30X10^{-6}$M ±0.3 (4)	ID_{25}= $8.4X10^{-6}$M ±1.6 (4)
Mouse Paired atria	ID_{25}= $9.30X10^{-7}$M ±3.2 (8)	ID_{25}= $3.70X10^{-7}$M ±1.5 (8)
Guinea Pig Unpaired atria	ID_{25}= $5.0X10^{-6}$M ±0.4 (6) ID_{50}= $3.42X10^{-6}$M ±0.5 (6)	ID_{25}= $2.52X10^{-6}$M ±0.4 (5)
Rabbit Unpaired atria	ID_{25}= $3.16X10^{-7}$M ±1.1 (4) ID_{50}= $1.50X10^{-6}$M ±0.7 (4)	ID_{25}= $4.01X10^{-7}$M ±1.4 (4)

As shown in Table 4.2, it can be seen that while the ID_{25} values obtained for nifedipine in the GP were in the 10^{-8}M range, those obtained in the mouse and rabbit were in the 10^{-7}M range. Hence this indicates that GP isolated atria are more sensitive to the calcium antagonistic action of nifedipine than mouse or rabbit artia. This may also suggest that the pacemaker function of the sinus node and the contractile activity of the cardiac cells in the guinea pig are more dependent on extracellular calcium than in mouse or rabbit.

In spite of the cost, it was therefore concluded from the results reported that the GP left atria (to monitor inotropic activity) and the spontaneously beating right atria (to monitor chronotropic activity) would be most appropriate for the testing of novel calcium channel blockers.

4.2 Effect of Nifedipine in Conscious Experimental Hypertensive and Normotensive Rats

The spontaneously hypertensive rat (SHR) was developed from the Japanese Wistar Kyoto strain (WKY) by Okamoto and Aoki (1963). It is widely used as an experimental hypertensive animal model in an attempt to understand the development, progression and maintenance of increased blood pressure. The present study was undertaken to establish whether the SHR could be used as a hypertensive animal model for the *in vivo* screening (antihypertensive effects) of novel calcium channel blockers. In particular the differential effects, if any, of nifedipine on the systolic blood pressure and heart rate in the conscious SHR and WKY animals was investigated.

The starting blood pressure of the SHR (183±7 mmHg, n=12) was significantly more elevated than the WKY (126 ±2 mmHg, n=12). Figure 4.1 and 4.2 depict the effect of an i.p. injection of vehicle and of 2, 4 and 8 mg/Kg nifedipine on the resting blood pressure of WKY and SHR rats respectively. Nifedipine produced a dose dependent decrease in blood pressure in both the SHR and WKY animals. However the response was more significant in the SHR. As exemplified in Table 4.3, after 20 min of a 4 mg/Kg i.p. injection of nifedipine the systolic blood pressure was lowered from 187±14 mmHg to 114±11 mmHg (39.0% decrease) in the SHR whereas in the WKY it decreased from 128±5 mmHg to 101±2 mmHg (21.1% decrease). A higher dose of 8 mg/Kg nifedipine

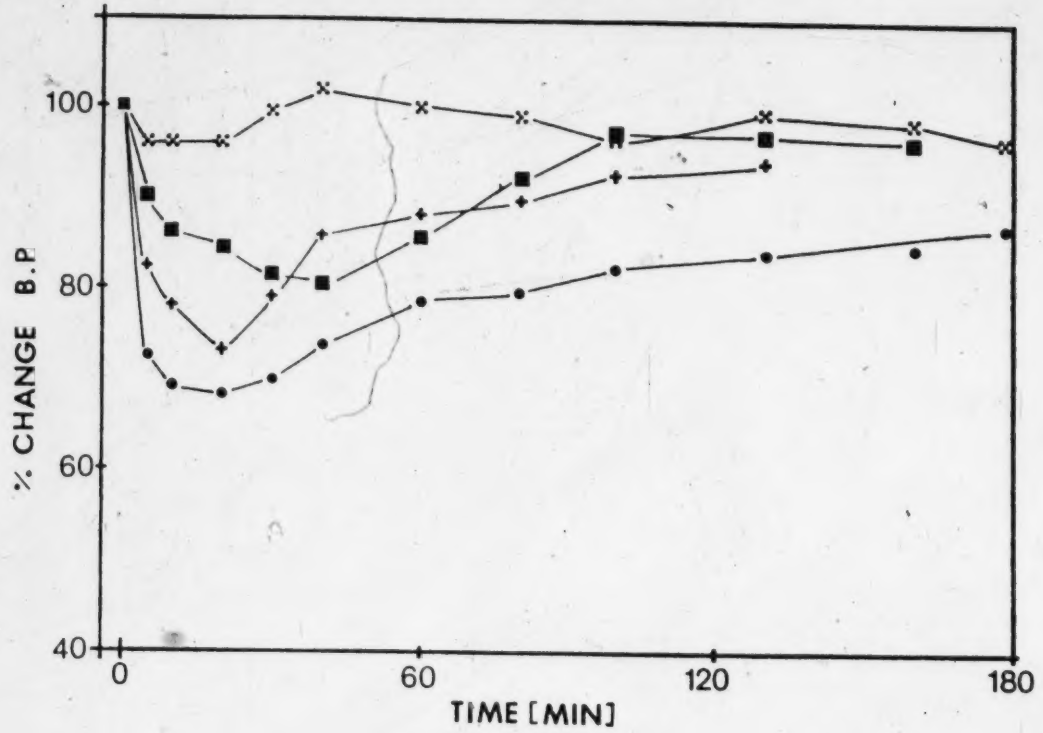


Figure 4.1 % Change in Blood Pressure as a function of time in the WKY rats after i.p. administration of vehicle (x), 2mg/Kg (▪), 4mg/Kg (+) and 8mg/Kg (•) Nifedipine. Each point represents the mean of 4 experiments. SEM bars are omitted for clarity of graph.

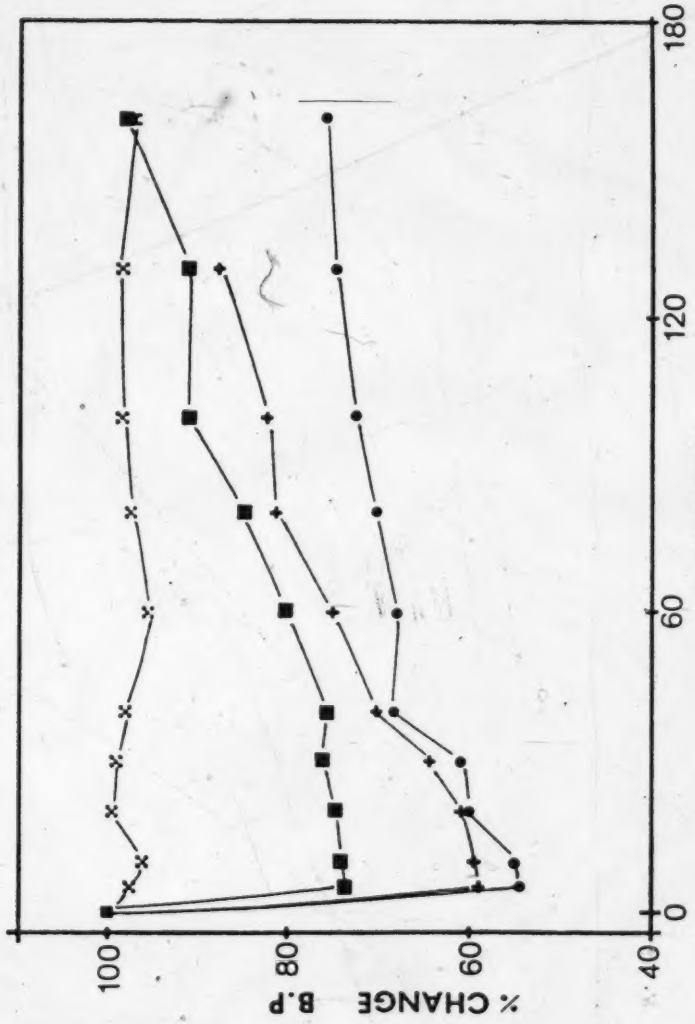


Figure 4.2 % Changein Blood Pressure as a function of time in the SHR rats after i.p. administration of vehicle (x), 2mg/Kg (■), 4mg/Kg (+) and 8mg/Kg (●) Nifedipine. Each point represents the mean of 4 experiments. SEM bars are omitted for clarity of graph.

produced a more sustained action in both types of animals.

Table 4.3

Effect of 4mg/Kg i.p. administration of nifedipine on the resting Blood Pressure of WKY and SHR rats. *Decrease in b.p significantly different from WKY at corresponding time ($P<0.05$).

TIME (min.)	BLOOD PRESSURE (mm Hg) $\bar{x} \pm$ SEM (n = 4) WKY	SHR
0	128 ± 5	187 ± 14
5	106 ± 3	109 ± 7*
10	98 ± 7	111 ± 10*
20	101 ± 2	114 ± 11*
30	101 ± 2	121 ± 11*
40	104 ± 4	131 ± 9
60	113 ± 4	137 ± 10
80	114 ± 6	150 ± 11
100	117 ± 6	152 ± 10
130	121 ± 4	159 ± 7

As shown in Figure 4.3 and 4.4, nifedipine produced increases in the heart rate of both SHR and WKY rats, this increase was not significantly different between the two types of animals. This effect is also observed in patients treated with nifedipine and has been attributed to a sympathetic reflex action in response to decreased arterial pressure.

The above results show that the hypertensive SHR model is more sensitive than the normotensive WKY rat to the antihypertensive action of the calcium channel antagonist nifedipine. For future *in vivo* antihypertensive screening of novel calcium channel blockers, testing on the SHR animals alone would be preferable.

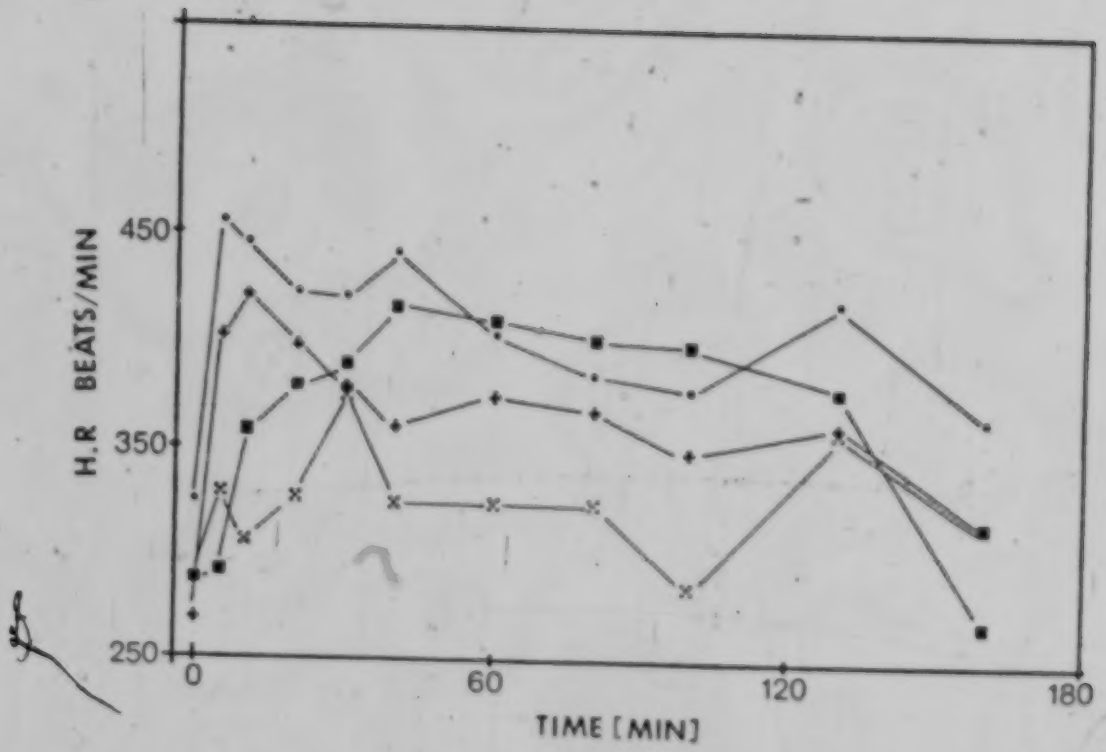


Figure 4.3 Typical responses obtained from single experiment: Changes in Heart Rate as a function of time in the WKY rats after i.p. administration of vehicle (x), 2mg/Kg (▪), 4mg/Kg (+) and 8mg/kg (•) Nifedipine.

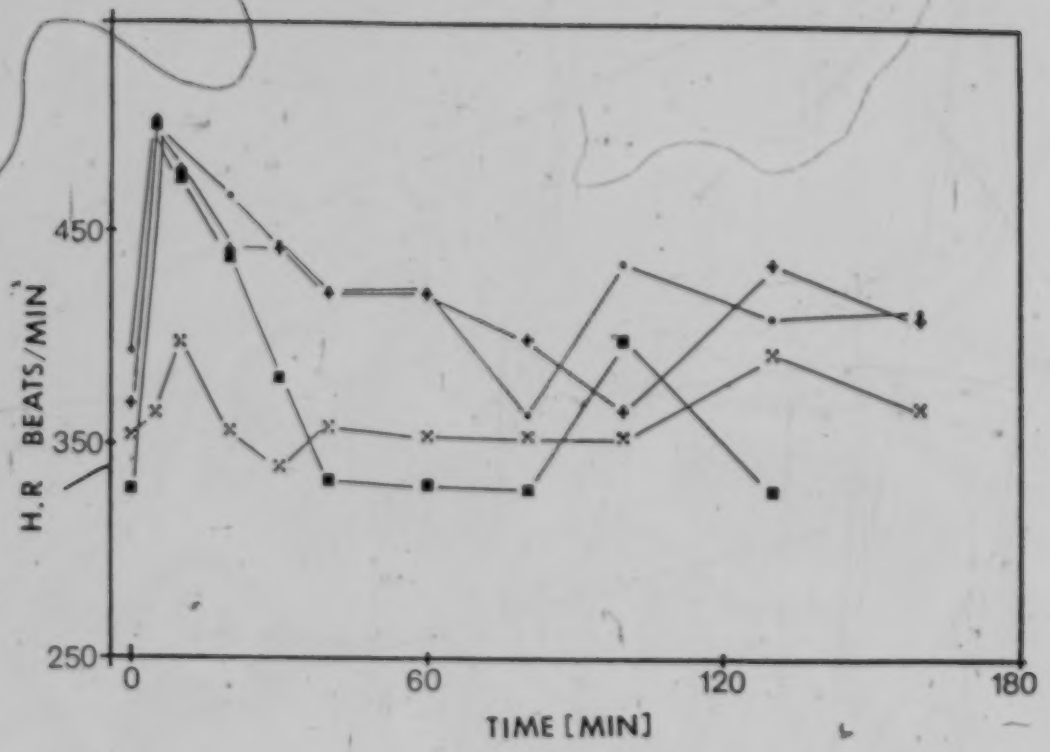


Figure 4.4 Typical responses obtained from single experiment: Changes in Heart Rate as a function of time in the SHR rats after i.p. administration of vehicle (x), 2mg/Kg (■), 4mg/Kg (+) and 8mg/Kg (•) Nifedipine.

These results appear to be compatible with reports that vascular smooth muscle responses to the physiological Ca^{++} ion are more sensitive in SHR than WKY (Harris *et al.*, 1983). However *in vitro* studies have resulted in conflicting observations about the differential effects of calcium channel blockers on isolated vascular tissues (Lederballe Pederson *et al.*, 1978; Nghiem *et al.*, 1982b; Pang and Sutter, 1981).

4.3 C4-Pyridinyl Nifedipine Analogs: Pharmacological Evaluation in GPILSM

As mentioned in section 3.4, the antagonist test drugs were incubated for 10 min prior to addition of the control agonist. A 10 min incubation time was arbitrarily chosen because it was previously found that the ID_{50} values of nifedipine determined by the protocol of 10 min and 30 min contact time were statistically not significant from each other. .The experimental method used to ascertain the preincubation time course for the test drug' s activity to reach equilibrium, involved challenging the resting GPILSM by a dose of the control agonist. When the tonic phase was reached and maintained, the antagonist test drug was then added; the tonic response was observed to decrease as the muscle relaxed. Equilibrium was indicated by the levelling off of the tonic response. Eight test drugs, independently chosen from each series of the compounds investigated in this present study were subjected to such an experimental protocol. They were observed to produce their equlibrium relaxant effect within 7 min. All compounds were therefore preincubated for 10 min using the experimental procedure described in the Methods section. Furthermore Hof and Vuorela (1983) after assessing 3 different methods of evaluating calcium antagonism activities, concluded that the method as adopted in this present study was preferable.

Table 4.4 shows the ID_{50} of a group of compounds in which the C4-o-nitro phenyl ring of nifedipine was replaced

by a 2'-, 3'- and 4'-pyridinyl ring, and the methyl ester substituents at the C3 and/or C5 positions replaced by other alkoxycarbonyl, N,N-dimethylaminoethyoxycarbonyl or CN groups.

Compounds 2, 6, 11 and 24 were evaluated for their inhibitory actions on both the muscarinic and KCl induced calcium dependent contractions. No statistical significant difference was observed between the ID_{50} values obtained through these two modes of agonist activation. This suggests that ID_{50} values obtained through muscarinic and KCl activation may be interchangeably compared.

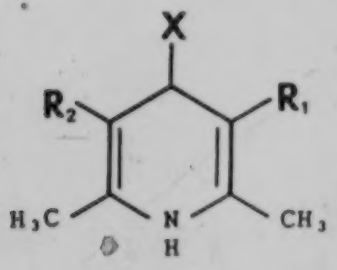
Compounds 1, 9 and 19 are structurally different from nifedipine in that the C4-o-nitro phenyl ring of nifedipine was replaced by a 2'-, 3'- and 4'-pyridinyl ring. This substitution provided compounds of significantly ($p<0.05$) lesser potency (10^{-4}M range activity as compared to 10^{-8}M for nifedipine).

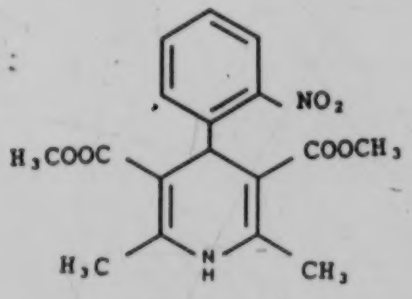
Among these pyridinyl compounds, analogs 1 to 6 with a 2'-pyridinyl ring were more active than the respective 3'-pyridinyl compounds 9 to 14, which in turn were more potent than the 4'-pyridinyl derivatives 19 to 23.

The size of the ester substituents at the C3 and C5 positions were increased from methoxy- to ethoxy-, iso-propoxy- and iso-butoxycarbonyl in each pyridinyl series. Such modification proved to be favourable for activity. As exemplified by the 2'-pyridinyl series, the order of activity was as follows: iso-butoxy analog 4 > iso-propoxy

Table 4.4

Calcium channel antagonist activity of C4-pyridinyl analogs in GPILSM. ID_{50}- concentration required to induce 50% inhibition of tonic muscarinic contration. Number in parentheses represents number of experiments. * indicates ID_{50} values obtained with KCl as control agonist instead of CD or CA.





NIFEDIPINE
ID_{50}=1.15X10^{-7}M ±0.1(28)

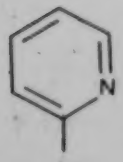
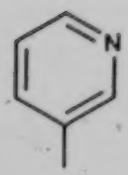
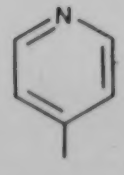
X	R_1	R_2	ID_{50} ±SEM	Cpd
2'-Pyridinyl 	COOMe	COOMe	2.32X10^{-7}M ±0.19(3)	1
	COOEt	COOEt	1.77X10^{-7}M ±0.03(3)	2
			2.64X10^{-7}M ±0.7(3*)	
	COOiPr	COOiPr	1.25X10^{-7}M ±0.2(3)	3
	COOiBu	COOiBu	9.36X10^{-8}M ±1.4(4)	4
	COOMe	COOiPr	9.56X10^{-8}M ±1.4(3)	5
	COOMe	COOiBu	4.10X10^{-8}M ±0.17(3)	6
			3.56X10^{-8}M ±0.5(3*)	
	COOMe	$COOCH_2N(CH_3)_2$	4.36X10^{-8}M ±0.5(4*)	7

Table 4.4 (cont')

X	R_1	R_2	ID_{50} ±SEM	Cpd
3'-Pyridinyl 	CN	CN	3.57X10⁻⁴M ±0.72(3)	8
	COOMe	COOMe	3.83X10⁻⁴M ±0.83(3)	9
	COOEt	COOEt	3.40X10⁻⁴M ±1.2(3)	10
	COOiPr	COOiPr	2.57X10⁻⁴M ±0.6(3)	11
			2.50X10⁻⁴M ±1.2(3*)	
	COOiBu	COOiBu	1.40X10⁻⁴M ±0.6(3)	12
	COOMe	COOiPr	9.70X10⁻⁴M ±4.67(4)	13
	COOMe	COOiBu	5.60X10⁻⁴M ±0.6(3)	14
	COOMe	$COOCH_2CH_2N(CH_3)_2$	3.34X10⁻⁴M ±0.5(4*)	15
	$COOCH_2CH_2N(CH_3)_2$	$COOCH_2CH_2N(CH_3)_2$	1.79X10⁻⁴M ±0.2(4*)	16
4'-Pyridinyl 	CN	CN	1.30X10⁻⁴M ±0.2(2)	17
	COOMe	CN	8.77X10⁻⁴M ±0.4(3)	18
	COOMe	COOMe	5.00X10⁻⁴M ±1.1(2)	19
	COOiPr	COOiPr	2.31X10⁻⁴M ±0.67(4)	20
	COOiBu	COOiBu	5.80X10⁻⁴M ±2.1(3)	21
	COOMe	COOiPr	5.60X10⁻⁴M ±1.6(3)	22
	COOMe	COOiBu	2.20X10⁻⁴M ±0.7(3)	23
	COOMe	$COOCH_2CH_2N(CH_3)_2$	5.05X10⁻⁴M ±0.8(3)	24
			5.13X10⁻⁴M ±0.4(4*)	

analog 3 > ethoxy analog 2 > methoxy analog 1. The same trend of results was also observed in the 3'- (9 to 12) and 4'- (19 to 21) pyridinyl series.

Compounds 3, 4, 11, 12, 16, 20 and 21 possessed identical esters at the C3 and C5 positions. Replacing one of these esters by a methoxycarbonyl resulted in compounds 5, 6, 13, 14, 15, 22 and 23 respectively. With the exception of 5 and 22, these latter compounds were found to be significantly ($p<0.05$) more active than their corresponding identical ester analogs.

Substitution of one or both of the methoxy groups at the C3 and C5 positions of compounds 1, 9 and 19 by N,N-dimethylaminoethoxy yielded the significantly ($p<0.05$) less active analogs 7, 15, 16 and 24.

Introduction of CN at either C3 and/or C5 positions of analogs 9 and 19 also produced compounds (8, 17 and 18) of significantly ($p<0.05$) less potency. Analog 17 which had both methoxycarbonyls replaced by CN was less active than compound 18 where only one ester was substituted.

The following summarizes the structure activity correlations of the C4-pyridinyl series of compounds:

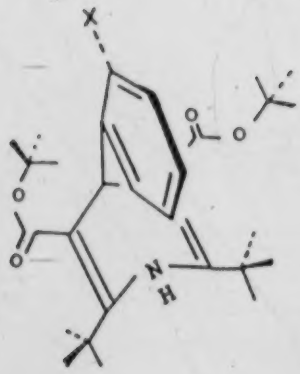
1. Replacement of the o-nitro phenyl ring of nifedipine by a pyridinyl decreased activity.
2. In terms of activity, 2'- > 3'- > 4'-pyridinyl analogs.
3. Increasing the size of the ester groups at the C3 and C5 positions enhanced activity.
4. Non-identical ester analogs were more potent than the

identical esters.

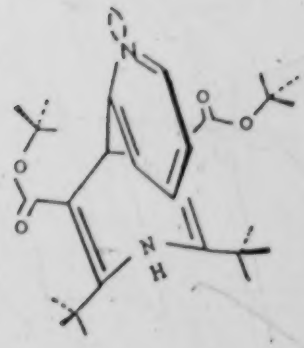
5. Substituting one or both of the ester groups by N,N-dimethylaminoethyl carboxylate significantly reduced activity.
6. Introduction of CN at either C3 and/or C5 positions decreased activity.

The 3-dimensional conformation of nifedipine (and related analogs) in the crystal state, has been determined by X-ray diffraction analysis (Fossheim *et al.*, 1982; Triggle *et al.*, 1980) and is depicted in Fig. 4.5. The dihydropyridine ring is seen to adopt a boat-like conformation with the C4 substituted phenyl ring above the boat in an axial orientation. Due to the non bonded interactions of the phenyl substituent and the C3 and C5 esters, the phenyl ring rotation is restricted so that the latter is perpendicularly positioned towards the 1,4-dihydropyridine ring in a N1-C4 symmetry plane. This may represent the ideal conformation for good calcium blockade activity.

As represented in Fig 4.5, C4-pyridinyl analogs are expected to adopt a similar conformation to nifedipine. Structurally both the C4-o-nitro phenyl ring of nifedipine and the C4-pyridinyl ring are planar. However nifedipine has an o-nitro substituent whereas the C4-pyridinyl compounds do not but instead have a lone pair of electrons on the C4-ring system. The decrease in activity seen when the o-nitro phenyl ring of nifedipine was replaced by a pyridinyl could therefore be attributed to the fact that the lone pair of



C4-phenyl



C4-pyridinyl

Figure 4.5 Comparison of the 3-dimensional structural representation of nifedipine and C4-2'-pyridinyl analog.

electrons on the pyridinyl ring have comparatively less steric effects than the o-nitro substituent of nifedipine. This would decrease the non bonded interactions between the C3, C4 and C5 substituents and also allow more free rotation of the C4-ring system. In fact Loev *et al.* (1974) also concluded that activity probably depended on the steric effect of the substituent on the C4 phenyl ring and that the electronic character was not important.

It is interesting to note that in parallel to the established SAR that the order of activity is ortho > meta > para substituted phenyl, it is observed here that 2'- > 3'- > 4'-pyridinyl, suggesting that the position of the nitrogen and lone pair of electrons in the pyridinyl ring is important for activity and that the pyridinyl ring is

partially bioisosteric with a substituted phenyl.

Analogs with bigger ester groups were found to be more potent, showing perhaps the importance of steric factors and/or lipophilicity properties of these compounds. Increasing the steric effects of the ester substituents would increase the non bonded interactions between the C3, C4 and C5 substituents, which is consistent with the fact that in the C4-pyridinyl series, non bonded interactions (as dictated by the distance between the lone pair of electrons and the C3 and C5 ester substituents) and activity increases in the same order of 2'- > 3'- > 4'-pyridinyl. It is also possible that the calcium channel blockers' receptor site consists of hydrophobic pockets to which the alkoxy substituents interact, and optimum activity is achieved through interaction of bulky ester substituents with these hydrophobic pockets.

The finding that activity increases with bigger ester groups is in total agreement with that of Loev and co-workers (1974) and Towart (1981a) but opposite to Rodenkirchen' s (1979) observations. However Rodenkirchen measured the negative inotropic effect of the dihydropyridines on papillary muscles while Loev looked at their hypotensive action on anaesthesized animals and Towart at relaxation of the GPILSM as used in this present study.

Replacement of iso-butoxy at either C3 and/or C5 positions by dimethylaminoethoxy was expected to enhance steric effects and hence increase activity, however such

substitution proved to be detrimental. A hypothetical explanation for this could be that the receptor site to which the ester substituent interacts with (or fits on) is a "cavity" site; the size of such cavity being ideal for the iso-butoxy group. In contrast nicardipine (2-(N-benzyl-N-methylamino)ethyl methyl-2,6-dimethyl,-4-(m-nitro phenyl)-1,4-dihydropyridine-3,5-dicarboxylate), which possess an even bulkier C3 ester substituent with a tertiary N, has been reported to possess good cerebral vasodilating effects and hence such substitution could be important for selective activity in the cerebral vascular tissues.

Compounds with non-identical substituents at the C3 and C5 positions were observed to be more active. This result confirms the finding reported by Iwanami and co-workers (1978). In these non-identical ester analogs, the carbon atom at the C4 position becomes a chiral center. In fact it is now known that such analogs exhibit stereoselectivity suggesting that dihydropyridine compounds bind to stereoselective sites to mediate their pharmacological effects (Towart, 1981a; Shibanuma *et al.*, 1980)

The decrease in activity seen when CN was substituted for the ester group could be attributed to the lesser steric effect of the CN substituent, and this would result in a decrease in non bonded interactions between the C3, C4 and C5 substituents, and also lead to freer rotation of the C4-ring system. The same SAR has previously been reported by Loev *et al.* (1974).

4.4 C4-Pyridinyl Nifedipine Analogs: Pharmacological Evaluation in GP Isolated Atria

As an extension of the study in the GPILSM which showed that increasing the size of the ester substituents at C3 and C5 positions increased activity, as opposed to the SAR reported by Rodenkirchen (1979) who found that in cat papillary muscles, activity decreases with more bulky esters, it was of interest to see what effect ester substitution of the C4-pyridinyl compounds, had on the contractile force (inotropic effect) and heart rate (chronotropic effect) of the GP isolated atria.

Table 4.5 shows the results of five C4-2'-pyridinyl derivatives, with ester substitution at the C3 and C5 positions. Chronotropic activity is seen to increase with larger ester groups (4>3>1). The influence on the inotropic effect was not the same, compound 1 had greater activity than compound 3, however they were both much less potent than 4. Replacing one iso-butoxy substituent of compound 4 by methoxy (i.e, the non-identical ester analog 6), did not appear to affect the cardiac activity significantly.

In parallel to what was observed in the GPILSM, dimethylaminoethoxy substitution of methoxy at the C3 position significantly ($p<0.05$) reduced activity (7 vs. 1).

It is therefore concluded that SAR for relaxant activity in GPILSM and chronotropic activity in GP isolated atria generally appears to be similar and thus increasing the size of the ester substituents tends to produce

Table 4.5

Calcium channel antagonist activity of C4-pyridinyl analogs in G.P isolated atria. ID_{50}- concentration required to induce 50% inhibition of the contractile activity of the left atria. ID_{25}- concentration required to induce 25% inhibition of the heart rate of the right spontaneously beating atria. Number in parentheses represents number of experiments. See Table 4.4 for structural details of compounds.

Left atria Inotropic E. ID_{50}	Right atria Chronotropic E. ID_{25}	Cpd
$1.67X10^{-4}M$ ±0.6 (4)	$3.13X10^{-4}M$ ±1.3 (4)	1
$3.34X10^{-4}M$ ±0.6 (3)	$4.51X10^{-3}M$ ±2.2 (3)	3
$9.37X10^{-4}M$ ±0.2 (3)	$1.98X10^{-3}M$ ±0.9 (3)	4
$6.72X10^{-4}M$ ±1.3 (4)	$4.67X10^{-3}M$ ±4.0 (4)	6
Conc. of $1.0X10^{-4}M$ to $5.0X10^{-4}$ produced no effect	$3.29X10^{-4}M$ ±0.8 (3)	7
$3.42X10^{-7}M$ ±0.5 (6)	$2.52X10^{-6}M$ ±0.4 (5)	Nifedipine

compounds of higher activity in GP isolated atria. These results are contrary to those of Rodenkirchen (1979) who found the opposite relationships on the inotropic activity in cat papillary muscle, and therefore deserve further consideration. These results may indicate a fundamental species difference in the nature of the receptors which influence inotropic activity in cat papillary muscle and the receptors which influence chronotropic activity in GP atria. Overall, more data are required to draw any clear correlation about the inotropic activity in the GP isolated atria.

4.5 C4-Dihydropyridinyl Nifedipine Analogs: Pharmacological evaluation in GPILSM

Compounds with a C4-(3' or 4')-[1'-substituted dihydropyridinyl] ring and ester group variations at C3 and C5 positions were also screened and the smooth muscle activities are reported in Table 4.6. The 1'-substituents were ' methoxy-, tert-butoxy- and phenoxycarbonyl. Compounds 25 to 44 consisted of a mixture of the 4-[3'-(1',2'-dihydropyridinyl)] and 4-[3'-(1',6'-dihydropyridinyl)] regioisomers. The separate activity of each regioisomer is not known.

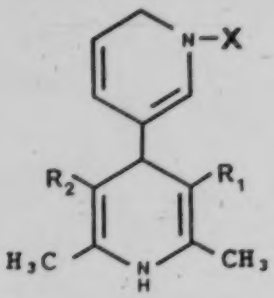
Replacement of the C4-o-nitro phenyl ring of nifedipine by a 3'- or 4'-dihydropyridinyl ring produced analogs 27, 33, 39 and 46 which had activity in the 10^{-6} to 10^{-7}M range as compared to nifedipine which was active at 10^{-8}M. Compounds 27 to 30 with a C4-3'-dihydropyridinyl were significantly ($p<0.05$) more potent than their corresponding 4'-dihydropyridinyl analogs 46 to 49.

A methoxycarbonyl substituent at the 1'-position of the C4-dihydropyridinyl ring, generally produced the most active compounds (27 to 32), whereas derivatives with tert-butoxy (33 to 38) and phenoxy (39 to 44) could not be differentiated in terms of their activities.

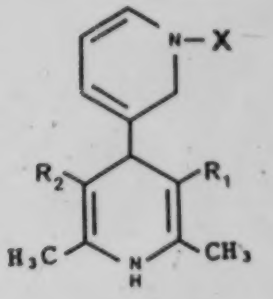
Compounds 25, 26 and 45 with a CN group at the C3 and/or C5 positions were found to be significantly ($p<0.05$) less active than compounds with a methoxycarbonyl substituent (27 and 46).

Table 4.6

Calcium channel antagonist activity of C4-dihydropyridinyl (dp) analogs in GPILSM. ID_{50}- concentration required to induce 50% inhibition of tonic muscarinic contraction. For comparison the ID_{50} of nifedipine = $1.15X10^{-9}M$. Number in parentheses represents number of experiments. * indicates ID_{50} values obtained with KCl as control agonist, instead of CD or CA.



C4-[3'-(1',6'-dp)] analog



C4-[3'-(1',2'-dp)] analog

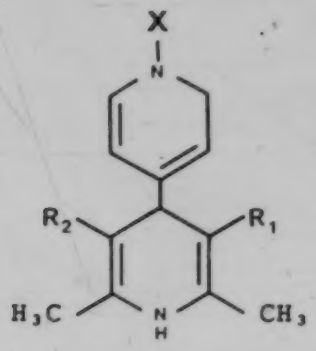
X	R_1	R_2	ID_{50} ±SEM	Cpd
COOMe	CN	CN	$3.39X10^{-9}M$ ±0.25(4)	25
COOMe	CN	COOMe	$5.16X10^{-9}M$ ±1.2(3)	26
			$3.70X10^{-9}M$ ±0.64(3*)	
COOMe	COOMe	COOMe	$5.89X10^{-9}M$ ±2.2(4)	27
			$9.60X10^{-9}M$ ±2.2(4*)	
COOMe	COOEt	COOEt	$1.8X10^{-9}M$ ±0.5(3)	28
COOMe	COOiPr	COOiPr	$1.31X10^{-9}M$ ±0.2(4*)	29
COOMe	COOiBu	COOiBu	$7.71X10^{-9}M$ ±1.7(3)	30
			$8.14X10^{-9}M$ ±1.3(4*)	
COOMe	COOMe	COOiPr	$1.58X10^{-9}M$ ±4.3(3)	31
			$8.54X10^{-9}M$ ±1.6(4*)	
COOMe	COOMe	COOiBu	$3.1X10^{-9}M$ ±0.7(4)	32

Table 4.6 (cont')

X	R_1	R_2	ID_{50} ±SEM	Cpd
COOPh	COOMe	COOMe	3.69X10⁻⁴M ±0.5(4)	33
			4.20X10⁻⁴M ±1.3(3*)	
COOPh	COOEt	COOEt	8.65X10⁻⁵M ±0.3(4)	34
			7.40X10⁻⁵M ±0.7(4*)	
COOPh	COOiPr	COOiPr	1.67X10⁻⁴M ±0.2(3)	35
			1.33X10⁻⁴M ±0.2(4*)	
COOPh	COOiBu	COOiBu	6.46X10⁻⁴M ±0.9(3)	36
			6.93X10⁻⁴M ±0.82(4*)	
COOPh	COOMe	COOiPr	9.0X10⁻⁵M ±1.7(4)	37
			9.18X10⁻⁵M ±1.4(4*)	
COOPh	COOMe	COOiBu	4.5X10⁻⁴M ±1.5(3)	38
			3.27X10⁻⁴M ±0.28(3*)	
COOtBu	COOMe	COOMe	5.77X10⁻⁴M ±0.3(4)	39
			4.77X10⁻⁴M ±0.6(4*)	
COOtBu	COOEt	COOEt	6.72X10⁻⁵M ±0.9(4*)	40
COOtBu	COOiPr	COOiPr	1.14X10⁻⁵M ±0.3(4)	41
COOtBu	COOiBu	COOiBu	3.42X10⁻⁴M ±0.4(3)	42
			3.72X10⁻⁴M ±0.8(4*)	
COOtBu	COOMe	COOiPr	2.37X10⁻⁵M ±0.3(4)	43
COOtBu	COOMe	COOiBu	1.28X10⁻⁵M ±0.1(4)	44

Table 4.6 (cont')

X	R_1	R_2	ID_{50} ±SEM	Cpd



C4-[4'-(1',2'-dp)] analog

COOMe	CN	CN	$6.75X10^{-9}M$ ±2.5(2)	45
COOMe	COOMe	COOMe	$7.47X10^{-9}M$ ±0.5(3)	46
			$6.87X10^{-9}M$ ±0.4(3*)	
COOMe	COOEt	COOEt	$2.43X10^{-9}M$ ±0.2(3)	47
			$1.97X10^{-9}M$ ±0.2(3*)	
COOMe	COOiPr	COOiPr	$1.01X10^{-9}M$ ±0.2(4)	48
COOMe	COOiBu	COOiBu	$1.72X10^{-9}M$ ±0.2(4)	49
			$1.81X10^{-9}M$ ±0.4(4*)	
COOMe	COOMe	COOiBu	$1.76X10^{-9}M$ ±0.5(4)	50
COOPh	COOMe	COOiBu	$8.10X10^{-9}M$ ±3.5(3)	51
COOPh	COOiBu	COOiBu	$5.10X10^{-9}M$ ±1.6(3)	52
COOtBu	COOiBu	COOiBu	$2.03X10^{-9}M$ ±0.5(4)	53
COOtBu	COOMe	COOiBu	$1.18X10^{-9}M$ ±0.2(4)	54

In the 3'- , 4'-[1'-methoxycarbonyl dihydropyridinyl] and 3'-[1'-tert-butoxycarbonyl dihydropyridinyl] groups of compounds, increasing the size of both the C3 and C5 esters from methoxy (27, 39 and 46) to ethoxy (28, 40 and 47) increased activity; a further increase to the corresponding iso-propoxy analogs 29, 41 and 48 further increased activity. However analogs 30, 42 and 49 which had iso-butoxy esters showed a decrease in activity with respect to analogs 29, 41 and 48 respectively.

In the 3'-[1'-phenoxycarbonyl dihydropyridinyl] group of compounds, derivative 33, with methoxy esters at the C3 and C5 positions was more active than the ethoxy ester analogs 34. However an increase of the size of the esters to iso-propoxy resulted in the less active compound 35; and any further increase to iso-butoxy (36) further reduced activity.

In contrast to the above case where both ester groups were increased, increasing the size of only one of the ester substituents (while the other one remained a methoxy-carbonyl) from methoxy (27, 33 and 39) to iso-propoxy (31, 37 and 43) and to iso-butoxy (32, 38 and 44) increased activity.

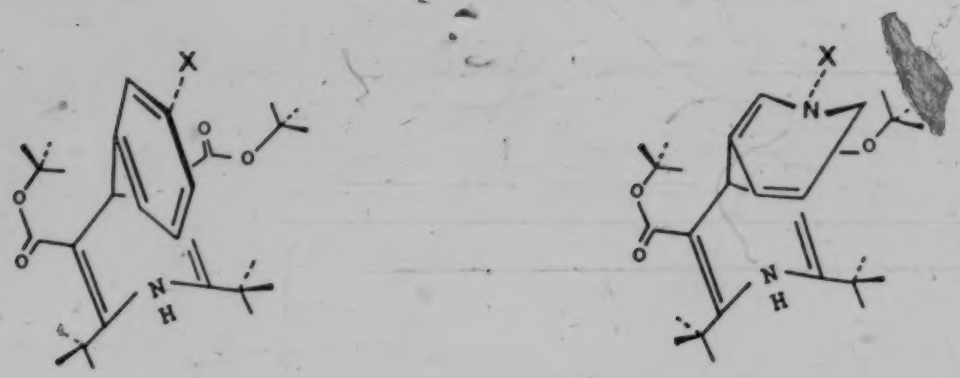
Compounds 30, 35, 36, 41 and 42 had identical iso-propoxy or iso-butoxy ester groups at the C3 and C5 positions. These compounds were significantly ($p < 0.05$) less active than their corresponding non-identical ester analogs 32, 37, 38, 43 and 44. In other cases, the identical ester derivatives

29, 49 and 53 were about equipotent as compared to their corresponding non-identical analogs 31, 50 and 54.

The following summarizes the determined SAR for this series of compounds:

1. Replacement of the 4-o-nitrophenyl ring of nifedipine by a 3' or 4'-dihydropyridinyl reduced activity.
2. Analogs with a 3'-dihydropyridinyl at the C4 position were more active than those with a 4'-dihydropyridinyl.
3. The order of activity for the substituent on the 1'-position of the C4-dihydropyridinyl was methoxy- > tert-butoxy- and phenoxycarbonyl.
4. Introduction of CN groups at either C3 or/and C5 positions reduced activity.
5. In the identical ester analogs, initial increase in the size of the esters increased activity, however any further increase in size reduced activity.
6. In the non-identical ester analogs, increase in the size of one of the ester while the other one was methoxy-carbonyl, increased activity.
7. Compounds with non-identical ester groups at the C3 and C5 positions were generally more potent than those with identical ones.

Figure 4.6 illustrates 3-dimensional representations of C4-meta-substituted phenyl and C4-(3'-[1'-substituted-dihydropyridinyl]) analogs. The decrease in activity which is observed when the o-nitrophenyl ring of nifedipine is replaced by a dihydropyridinyl, could be attributed to the



C4-phenyl

C4-dihydropyridinyl

Figure 4.6 Comparison of the 3-dimensional structural representation of C4-phenyl and C4-dihydropyridinyl analogs.

more puckered conformation of the latter ring system, as compared to the planar phenyl ring of nifedipine. This suggests that planarity of the C4-ring substituent is important for good calcium antagonist activity.

Compounds with either methoxy-, tert-butoxy- or phenoxycarbonyl substituents on the 1'-position of the C4-dihydropyridinyl ring were tested to assess the steric effect of these substituents on activity. In terms of steric properties, tert-butoxy and phenoxy substituents are bulkier than methoxy. Since the smaller methoxy substituent yielded compounds of better acivity than the tert-butoxy and phenoxy analogs, it appears that substituents of smaller size on the C4-dihydropyridinyl ring are optimal for calcium channel blocking activity.

Structure activity correlations #2, 4, 6 and 7 are consistent with those obtained in the C4-pyridinyl series. However it is very interesting to note that when both the ester substituents at C3 and C5 positions were increased in size, highest activity was obtained with an "optimum" size of ester substituent. Any further increase in size had a detrimental effect on activity.

Interestingly, the above relationship was not present when only one ester group was increased (the other one being methoxycarbonyl) and also was absent in the C4-pyridinyl series. Since pyridinyl and dihydropyridinyl rings are conformationally different, this could mean that binding of that part of the ring system to the receptor site may differ, and this could indirectly affect (e.g. through steric hindrance) the interaction of the ester substituent with its binding site and hence a different SAR for the ester groups of the dihydropyridinyl series of compounds. Also non-identical ester analogs possess a chiral center which may well be important for the increased activity observed.

As mentioned in the Methods section, after inhibition by the test drugs is obtained, the original control responses were usually regained within about 30 min. However seven compounds in this series of C4-dihydropyridinyl analogs (30,35,36,42,49,52,53) were observed to have a longer duration of action, as judged by the longer time required for the recovery of control responses. Figure 4.7

shows a typical experiment where agonist control doses were repeated after inhibition by the antagonist test drug was obtained. As exemplified by nifedipine and compound 44, the original control responses were regained within about 30 mins. However for compound 42, after three and a half hours the response was only 53% of the original control response. Compound 42 possesses identical iso-butoxycarbonyl esters at the C3 and C5 positions, while compound 44 has iso-butoxy- and a methoxycarbonyl substituents. In fact one common property shared by these compounds is that they possess bulky identical ester substituents at the C3 and C5 positions, which may allow them to bind more strongly to the channel protein.

In this series, 15 of the 30 novel dihydropyridinyl analogs were evaluated on the GPILSM for their inhibitory effect on both the muscarinic and KCl-depolarization calcium dependent contractions. For each analog, an unpaired t-test was performed between the ID_{50} values obtained through the 2 modes of activation, i.e muscarinic and KCl-depolarization. In all cases, no statistical significance was observed (p 0.5). Hence in the GPILSM, muscarinic and KCl induced contractions are equally sensitive to the action of dihydropyridine calcium channel blockers. This suggests that muscarinic agonists and KCl induce calcium entry through similar mechanisms. This may also suggests that muscarinic agonists on binding to their receptors produce depolarization and therefore induce calcium entry through POC similar

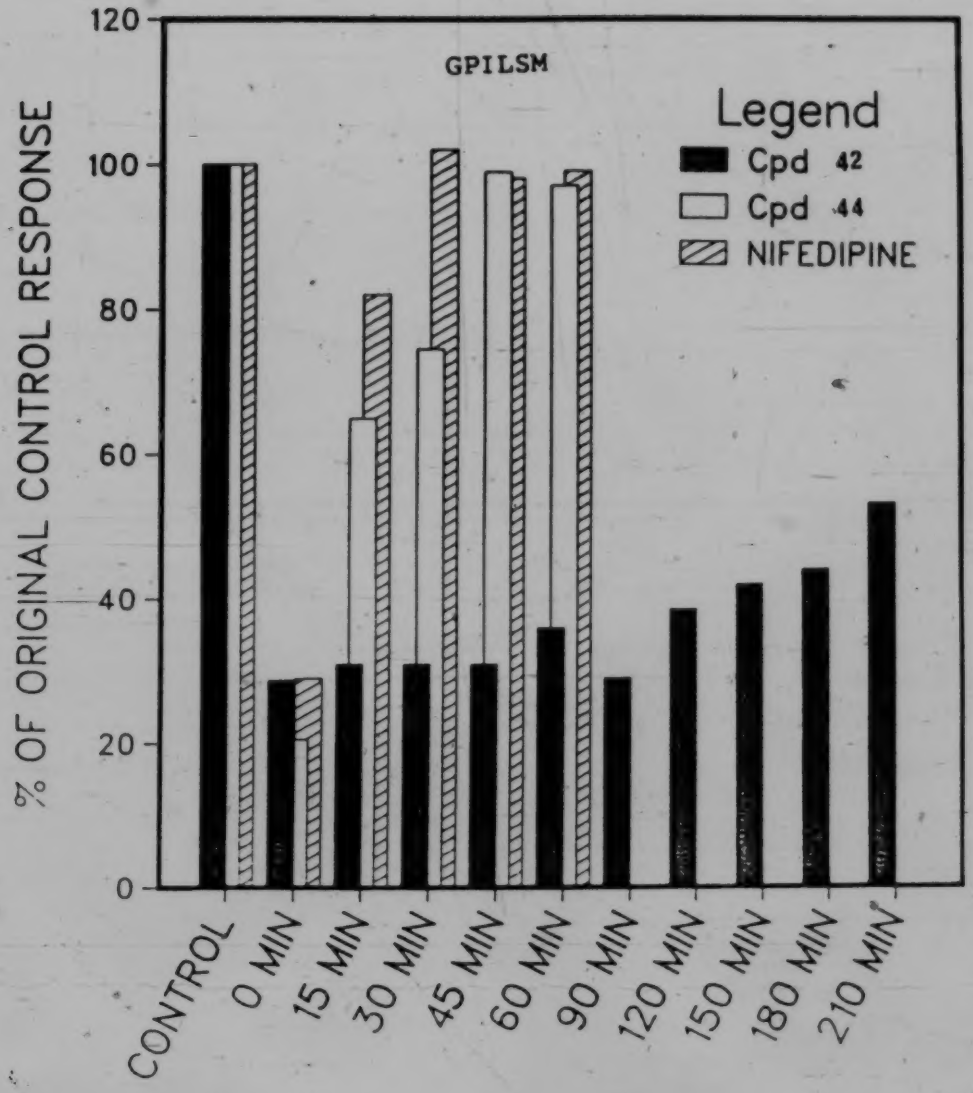


Figure 4.7 Recovery of agonist control responses after inhibition by nifedipine ($2.67X10^{-9}M$), compound 44 ($2.90X10^{-7}M$) and 42 ($1.14X10^{-5}M$). Responses at 0 min represents responses obtained in presence of the antagonist previously incubated for 10 min, thereafter the control doses were repeated every 15 min.

to KCl-depolarization. However, since electrophysiological data are not available for the GPILSM, the above results may also indicate that ROC and POC are equally susceptible to the action of dihydropyridine calcium channel blockers in the GPILSM.

The spontaneously hypertensive rat (SHR) which was shown to be a good animal model for the screening of anti-hypertensive action of calcium channel blockers (section 4.2), was used to further evaluate compound 32. Figure 4.8 illustrates an experiment where an i.p. injection (4mg/Kg) of the novel dihydropyridinyl analog 32 was administered to conscious SHR rats.

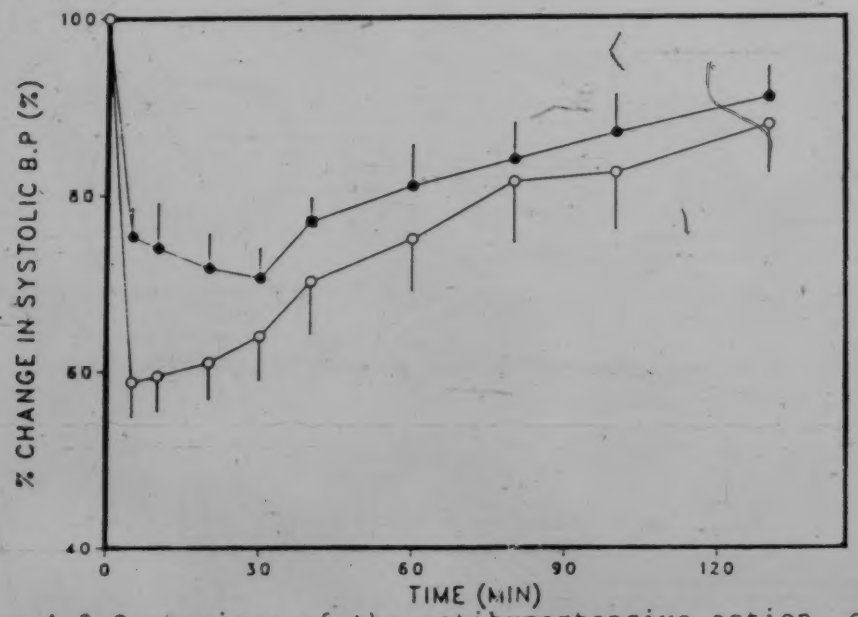


Figure 4.8 Comparison of the antihypertensive action of an i.p. administration of 4mg/Kg compound 32 (•) and nifedipine (o) on conscious SHR rats. Each point represents the mean of 3 (•) and 4 (o) experiments ± SEM.

Compound 32 is seen to lower the blood pressure by 30% after 30 min of injection, and therefore has antihypertensive action. As compared to nifedipine, compound 32's antihypertensive effect was less pronounced, but nonetheless significant. This was also comparable to the relaxant action in GPILSM, where the ID_{50} of analog 32 was $3.1X10^{-8}M$ as compared to $1.15X10^{-8}M$ for nifedipine.

4.6 C4-Dihydropyridinyl Nifedipine Analogs: Pharmacological evaluation in GP isolated atria

Table 4.7 displays the cardiac activities of seven C4-dihydropyridinyl analogs. The insolubility of some analogs at higher concentration did not allow the determination of the negative inotropic effect (ID_{50} values) of these compounds in the left atrium.

Similar to the SAR observed in the GPILSM, compounds with a methoxy substituent at the 1'- position of the dihydropyridine were more potent than those with phenoxy or tert-butoxy on the chronotropic effet (27>33,39). Hence for chronotropic activity in the GP isolated atria, a smaller substituent on the 1'-position of the dihydropyridine ring is optimal. Such a relationship may also exist for the inotropic effect since compound 27 was more potent than compound 39.

Increasing the size of the ester groups at the C3 and C5 positions from methoxy- to iso-butoxycarbonyl did not affect the chronotropic activity significantly, and this is therefore equivalent to the relaxant effect in GPILSM (27,39 vs. 30,42). Furthermore compound 44 which had non-identical esters at the C3 and C5 positions was significantly more active than compound 42 and 39 which had identical ones.

Compound 46 with a C4-4'-dihydropyridinyl ring was less potent than the isomeric 3'-dihydropyridinyl analog 27, in reducing chronotropic as well as inotropic activity. These two compounds were less active than nifedipine. These

Table 4.7

Calcium channel antagonist activity of C4-dihydropyridinyl (dp) analogs in G.P isolated atria. ID_{50}- concentration required to induce 50% inhibition of the contractile activity of the left atria. ID_{25}- concentration required to induce 25% inhibition of the heart rate of the right spontaneously beating atria. Number in parentheses represents number of experiments.

Left atria Inotropic E. ID_{50}	Right atria Chronotropic E. ID_{25}	Cpd
$3.87X10^{-4}M$ ±1.7 (4)	$9.87X10^{-4}M$ ±2.5 (5)	27
insoluble	$3.10X10^{-4}M$ ±0.4 (3)	30
insoluble	$1.63X10^{-5}M$ ±0.6 (4)	33
$1.55X10^{-4}M$ ±0.4 (5)	$1.24X10^{-5}M$ ±0.4 (5)	39
insoluble	$1.10X10^{-5}M$ ±0.9 (3)	42
$4.08X10^{-4}$ ±1.6 (3)	$2.25X10^{-5}$ ±1.4 (3)	44
Conc. of $1.0X10^{-5}M$ to $5.0X10^{-4}M$ produced no effect	$1.28X10^{-5}M$ ±0.2 (4)	46
$3.42X10^{-5}M$ ±0.5 (6)	$2.52X10^{-4}M$ ±0.4 (5)	Nifedipine

findings are equivalent to the relaxant activity in GPILSM.

Hence in the dihydropyridinyl series of compounds, similar SAR were obtained in both the GP isolated artia and in the GPILSM, suggesting that the receptors in these two preparations possess some identical properties.

4.7 C4-Tetrahydropyridinyl Nifedipine Analogs: Pharmacological evaluation in GPILSM

Table 4.8 shows the ID_{50} of compounds with a 5', 2' or 4'-(1'-methyl-1',2',3',6'-tetrahydropyridinyl) at the C4 position of nifedipine, and the ester substituents at the C3 and C5 positions being either methoxy- or ethoxycarbonyl.

Replacement of the C4-o-nitro phenyl ring of nifedipine by a tetrahydropyridinyl yielded the significantly (p 0.05) less active compounds 55, 57 and 59. Further modification of these compounds led to a significant (p 0.05) increase in activity, this was achieved by substituting the methoxycarbonyl at the C3 and C5 positions by ethoxycarbonyl (56, 58 and 60).

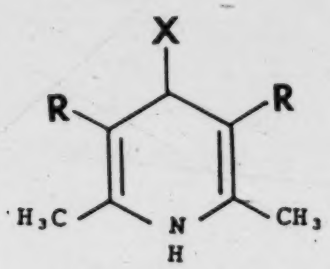
In the methoxycarbonyl series, the 5'-tetrahydropyridinyl analog 55 was more potent than the 2'-tetrahydropyridinyl analog 57, which in turn was more potent than the 4'-tetrahydropyridinyl analog 59. Among the ethoxycarbonyl derivatives, the 5'-tetrahydropyridinyl compound 56 was more active than the 2'-tetrahydropyridinyl analog 58, however the latter was less active than the 4'-tetrahydropyridinyl analog 60.

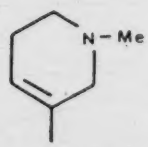
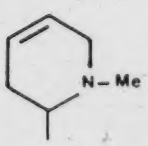
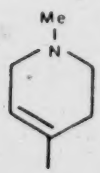
The SAR of the C4-tetrahydropyridinyl analogs are summarized as follows:

1. Substitution of the o-nitrophenyl ring of nifedipine by a tetrahydropyridinyl significantly reduced activity.
2. Activity was enhanced when the methoxycarbonyl substituents at the C3 and C5 positions were replaced by

Table 4.8

Calcium channel antagonist activity of C4-tetrahydropyridinyl (tp) analogs in GPILSM. ID_{50}- concentration required to induce 50% inhibition of tonic muscarinic contraction. For comparison the ID_{50} of nifedipine = $1.15X10^{-9}M$. Number in parentheses represents number of experiments. * indicates ID_{50} values obtained with KCl as control agonist, instead of CD or CA.



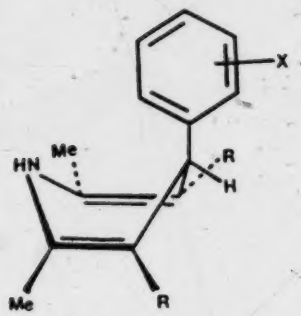
X	R	ID_{50} ±SEM	Cpd
5'-tp			
	COOMe	$3.43X10^{-9}M$ ±0.5(3)	55
	COOEt	$1.10X10^{-9}M$ ±0.7(3)	56
2'-tp			
	COOMe	$6.47X10^{-8}M$ ±0.24(3)	57
	COOEt	$1.30X10^{-8}M$ ±0.05(3)	58
		$5.15X10^{-9}M$ ±0.70(3*)	
4'-tp			
	COOMe	$1.81X10^{-9}M$ ±0.5(3)	59
	COOEt	$4.96X10^{-9}M$ ±1.33(3)	60

ethoxycarbonyl.

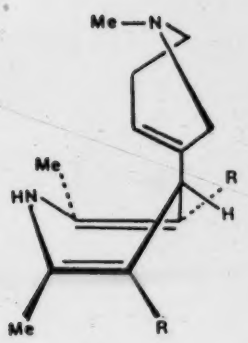
3. In the methoxycarbonyl series, the order of activity was 5'- > 2'- > 4'-tetrahydropyridinyl, while in the ethoxycarbonyl 5'- > 4'- > 2'-tetrahydropyridinyl.

As depicted in Fig. 4.9, the tetrahydropyridinyl is a very distorted puckered ring as compared to a planar phenyl or pyridinyl ring. The fact that pyridinyl and substituted phenyl analogs are significantly more active than tetrahydropyridinyl compounds may imply that conformation (planar ring system being ideal) and/or degree of unsaturation of the C4-ring substituent is essential for good calcium antagonistic activity in GPILSM. This hypothesis is substantiated by the fact that dihydropyridinyl analogs possess better activity than tetrahydropyridinyl ones, since a dihydropyridinyl ring is less puckered and less saturated than a tetrahydropyridinyl.

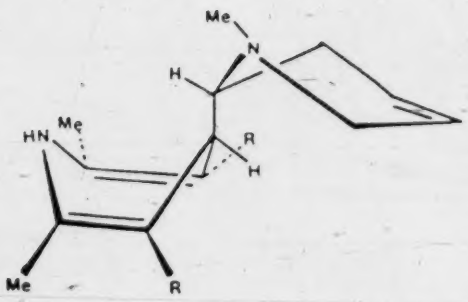
At the C4 position, the order of activity was ortho > meta > para-substituted phenyl analogs, similarly 2'- > 3'- > 4'-pyridinyl and 3'- > 4'-dihydropyridinyl. However the tetrahydropyridinyl series of compounds did not follow this same order of activity (see SAR #3 above). This may partially be explained by Fig. 4.9 which illustrates and compares the possible orientation of the 5'-, 2'- and 4'-tetrahydropyridinyl ring to the boat shaped 1,4-dihydropyridine ring. The 1,4-dihydropyridine ring of the 4'- and 5'-tetrahydropyridinyl analogs is linked to the tetrahydropyridinyl ring through an sp^2 hybridised carbon and



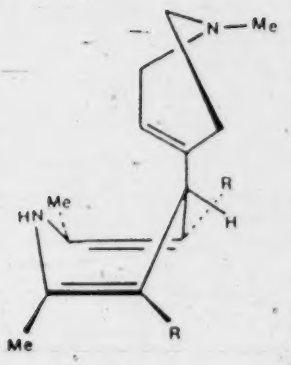
C4-phenyl



5'-tp



2'-tp



4'-tp

Figure 4.9 Comparison of the 3-dimensional structural representation of C4-phenyl and C4-tetrahydropyridinyl analogs.

therefore may be expected to adopt a perpendicular orientation as seen in nifedipine and C4-pyridinyl analogs. On the other hand, the 1,4-dihydropyridine ring of the 2'-tetrahydropyridinyl analogs is attached to an sp^3 hybridised carbon and thus may possibly exist in a co-planar orientation to the tetrahydropyridinyl ring. If this is so, the result that the order of activity is 56 > 60 > 58 suggest that perpendicular orientation of the C4 ring substituent to the 1,4-dihydropyridine ring is necessary for good calcium channel blockade activity. However this does not explain why compound 57 was about twice as potent as compound 59.

Similar to the C4-pyridinyl and and C4-dihydropyridinyl series, in this series of C4-tetrahydropyridinyl compounds, ethoxycarbonyl substituents at the C3 and C5 positions in place of a methoxycarbonyl enhanced activity.

4.8 Substitution of the 1,4-Dihydropyridine Ring of Nifedipine by 1,2-Dihydropyridine: Pharmacological Evaluation in GPILSM

Compounds with the 1,4-dihydropyridine ring of nifedipine replaced by 1,2-dihydropyridine, were also screened and the results are listed in Table 4.9. Up until now, no work on the pharmacological screening of such compounds has been reported. Hence from the test results, it was possible to assess in this series of compounds, the effect of ortho, meta and para substitution on the C4-phenyl ring by NO_2 and CF_3 groups, as well as the effect of other ester groups at the C3 and C5 positions.

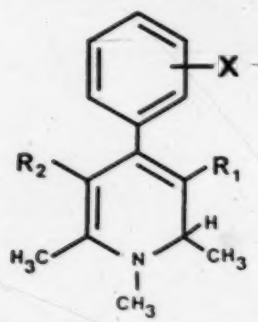
Since all the 1,2-dihydropyridine compounds tested were N-methylated, the N-methylated derivative of nifedipine (1,2,3,5,6-pentamethyl-4-(2-nitro phenyl)-1,4-dihydropyridine-3,5-dicarboxylates) as pictured in Fig. 4.10, was also screened in the GPILSM and was found to be less potent. This is in agreement with what has been reported previously (Iwanami *et al.*, 1978).

With respect to compound 73, analog 67 possess a 1,2-dihydropyridine instead of the classical 1,4-dihydropyridine ring. This substitution resulted in a 100 fold decrease in activity.

Compounds 68 and 69 have an unsubstituted phenyl ring at the C4 position. In most cases, substitution on the phenyl ring significantly ($p < 0.05$) reduced activity (61, 63, 65, 66 and 67). In other cases the resulting compounds (62

Table 4.9

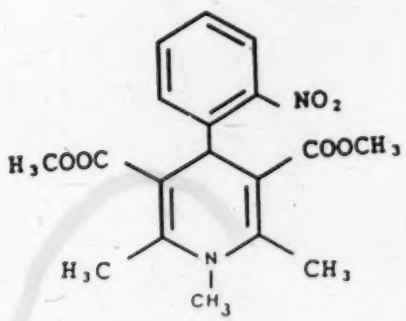
Calcium channel antagonist activity of 1,2-dihydropyridine analogs in GPILSM. ID_{50}- concentration required to induce 50% inhibition of tonic muscarinic contraction. For comparison the ID_{50} of nifedipine = $1.15X10^{-8}$M. Number in parentheses represents number of experiments. * indicates ID_{50} values obtained with KCl as control agonist, instead of CD or CA.



X	R_1	R_2	ID_{50} ±SEM	Cpd
p-CF_3	COOMe	COOMe	$8.92X10^{-4}$M ±1.06(4)	61
m-CF_3	COOMe	COOMe	$3.50X10^{-4}$M ±0.8(3)	62
o-CF_3	COOMe	COOMe	$1.20X10^{-5}$M ±0.1(3)	63
p-NO_2	COOEt	COOEt	$5.71X10^{-4}$M ±0.84(3)	64
p-NO_2	COOMe	COOMe	$2.35X10^{-5}$M ±0.68(4)	65
			$2.05X10^{-5}$M ±0.01(3*)	
m-NO_2	COOMe	COOMe	$7.90X10^{-4}$M ±1.60(2)	66
o-NO_2	COOMe	COOMe	$2.52X10^{-5}$M ±0.23(4)	67
H	COOMe	COOMe	$2.15X10^{-4}$M ±0.5(2)	68
H	COOEt	COOEt	$6.84X10^{-4}$M ±0.16(3)	69
o-NO_2	COOiPr	COOiPr	$5.73X10^{-4}$M ±0.30(7)	70
o-NO_2	COOMe	COOiPr	$2.00X10^{-5}$M ±0.14(7)	71
o-NO_2	COOiPr	COOMe	$1.05X10^{-5}$M ±0.11(7)	72

and 64) were about equipotent to their respective unsubstituted phenyl analogs.

The meta-substituted phenyl analogs 62 and 66 were more potent than the para-substituted ones (61 and 65). These latter compounds were in turn more active than the ortho-substituted phenyl analogs 63 and 67.



Cpd 73

$ID_{50}=2.41X10^{-7}M \pm 0.13(4)$

Figure 4.10 Structure and activity of the N-methylated derivative of nifedipine. Data are given as mean ± SEM. ID_{50}- concentration required to induce 50% inhibition of muscarinic contraction. n is number of experiments.

The CF_3-substituted phenyl analogs 61, 62 and 63 were significantly ($p<0.05$) more active than the corresponding NO_2-substituted ones (65, 66 and 67). Compound 70 which has identical iso-propoxy esters at both C3 and C5 positions was significantly ($p<0.05$) more active than the non-identical ester analogs 71 and 72.

The following summarizes the SAR of the 1,2-dihydropyridine analogs:

1. Substitution by a 1,2 dihydropyridine ring resulted in compounds of significantly reduced activity.
2. The presence of a substituent on the C4 phenyl ring either decreased or did not affect activity.
3. The order of activity was meta > para > ortho-substituted phenyl analogs.
4. CF_3 substituted phenyl analogs had better activity than NO_2 substituted ones.
5. Compounds with non-identical ester substituents were less potent than those with the same ester groups.

SAR #2, 3 and 5 as described above, do not conform to the previously established structure activity correlations which show that C4-substituted phenyl analogs are more active than unsubstituted ones; the order of activity is ortho > meta > para-substituted phenyl analogs; compounds with non-identical ester groups are more potent than those with identical ones. Hence the requirements for optimum activity differs significantly between the 1,2- and 1,4-dihydropyridine calcium channel blockers.

This change in activity requirements is probably brought about by the significant difference that exists between the conformation of these two groups of compounds (Fig. 4.11). Thus the 1,2-dihydropyridinyl ring does not exist in a boat shaped conformation. It is linked to the C4-phenyl ring by an sp^2 hybridized C4 carbon which therefore should make the 1,2-dihydropyridine ring co-planar with the C4-phenyl ring (as opposed to a perpendicular

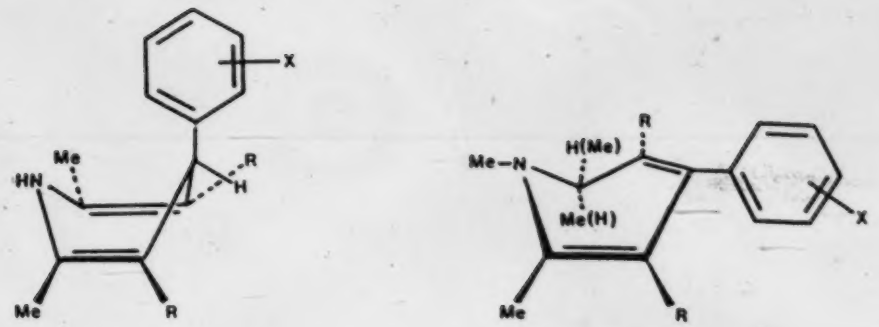


Figure 4.11 3-dimensional stuctural representation of 1,4- and 1,2-dihydropyridine analogs.

orientation in 1,4-dihydropyridine analogs). Another factor is that the C2 carbon of the 1,2-dihydropyridine compounds is a chiral center, whereas in the 1,4-dihydropyridine analogs the C4 carbon is chiral if the ester substituents are different. The separate activity of the 1,2-dihydropyridine enantiomers are not known since they were not separated.

Due to these conformational differences, the orientation of the C3, C4 and C5 substituents of the 1,2- and 1,4-dihydropyridine analogs with the receptor sites may well be different; this could therefore lead to differences in the way that these two groups of compounds fit and interact with the receptor(s) and hence they may possibly have some different activity requirements.

SAR #4 was also observed by Rodenkirchen *et al.* (1979) in the 1,4-dihydropyridine series of compounds. As a matter of fact, of all the SAR derived for the 1,2-dihydropyridine series of analogs, SAR #4 was the only one that was common to both the 1,4- and 1,2-dihydropyridine series.

4.9 C4-Pyridinyl Analogs of Bay K8644: Pharmacological Evaluation in GPILSM and GP Isolated Atria

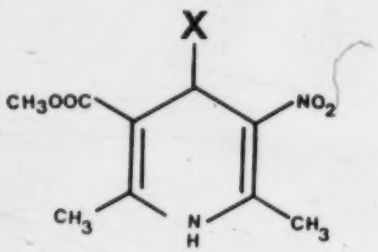
Bay K8644 and CGP 28392 are two nifedipine analogs that have been reported to be calcium agonists, i.e they exhibit positive inotropic and positive chronotropic effects and induce smooth muscle contraction. Table 4.10 shows the pharmacological profile of three analogs of Bay K8644 where the C4-trifluoromethylphenyl ring was replaced by 2'-, 3'- or 4'-pyridine ring (2,6-dimethyl-5-nitro-(2'-, 3'-, 4'-dihydropyridine-3-carboxylate).

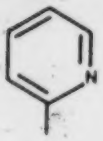
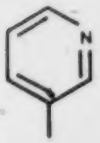
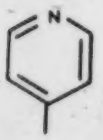
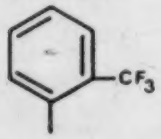
The three test compounds were screened in both the GPILSM and the GP isolated atria preparations. The 2'-pyridinyl analog caused relaxation of the GPILSM with an ID_{50} of $2.12X10^{-4}M$, it had -ve chronotropic effect (as shown by an ID_{25} of $2.62X10^{-5}$ on the right spontaneously beating heart), but in cumulative doses from $1.0X10^{-7}M$ up to $8.0X10^{-4}M$ failed to produce any significant change in the inotropic activity of the isolated left atria.

As opposed to the 2'-pyridinyl analog, the 3'- and 4'-pyridinyl analogs of Bay K8644 showed calcium agonist properties; they produced +ve inotropic and +ve chronotropic effects on the isolated atria preparations, and also induced contraction in the GPILSM (Fig 4.12a and b). In addition, compound 76 was observed on all occasions, to produce cardiac disturbances (ectopic beats) on isolated left atria at higher concentrations (Fig 4.12c).

Table 4.10

Pharmacological profile of C4-2'-, 3'- and 4'-pyridinyl analogs of Bay K8644. ID_{50}- concentration required to induce 50% inhibition of tonic muscarinic contraction in GPILSM. ID_{25}- concentration required to induce 25% inhibition of the heart rate of the right spontaneously beating atria. ED_{50}- concentration required to produce 50% of the maximal response, i.e contractile activity in GPILSM, contractile activity in left atrium and heart rate in right atrium.



X	GPILSM	Left atria Inotropic E.	Right atria Chronotropic E.
Cpd 74 	ID_{50}= $2.12X10^{-4}$M ±0.7 (3)	Conc. of $1.0X10^{-7}$M to $8.0X10^{-4}$M produced no effect	ID_{25}= $2.6X10^{-5}$M ±0.5 (4)
Cpd 75 	ED_{50}= $6.72X10^{-5}$M ±1.1 (5)	ED_{50}= $7.0X10^{-5}$M ±1.4 (4)	ED_{50}= $1.09X10^{-4}$M ±0.3 (5)
Cpd 76 	ED_{50}= $2.67X10^{-5}$M ±0.7 (6)	ED_{50}= $4.85X10^{-5}$M ±0.3 (4)	ED_{50}= $1.12X10^{-4}$M ±0.2 (4)
Bay K8644 	Not done	ED_{50}= $2.28X10^{-7}$M ±0.5 (4)	ED_{50}= $1.89X10^{-4}$M ±10.5 (4)

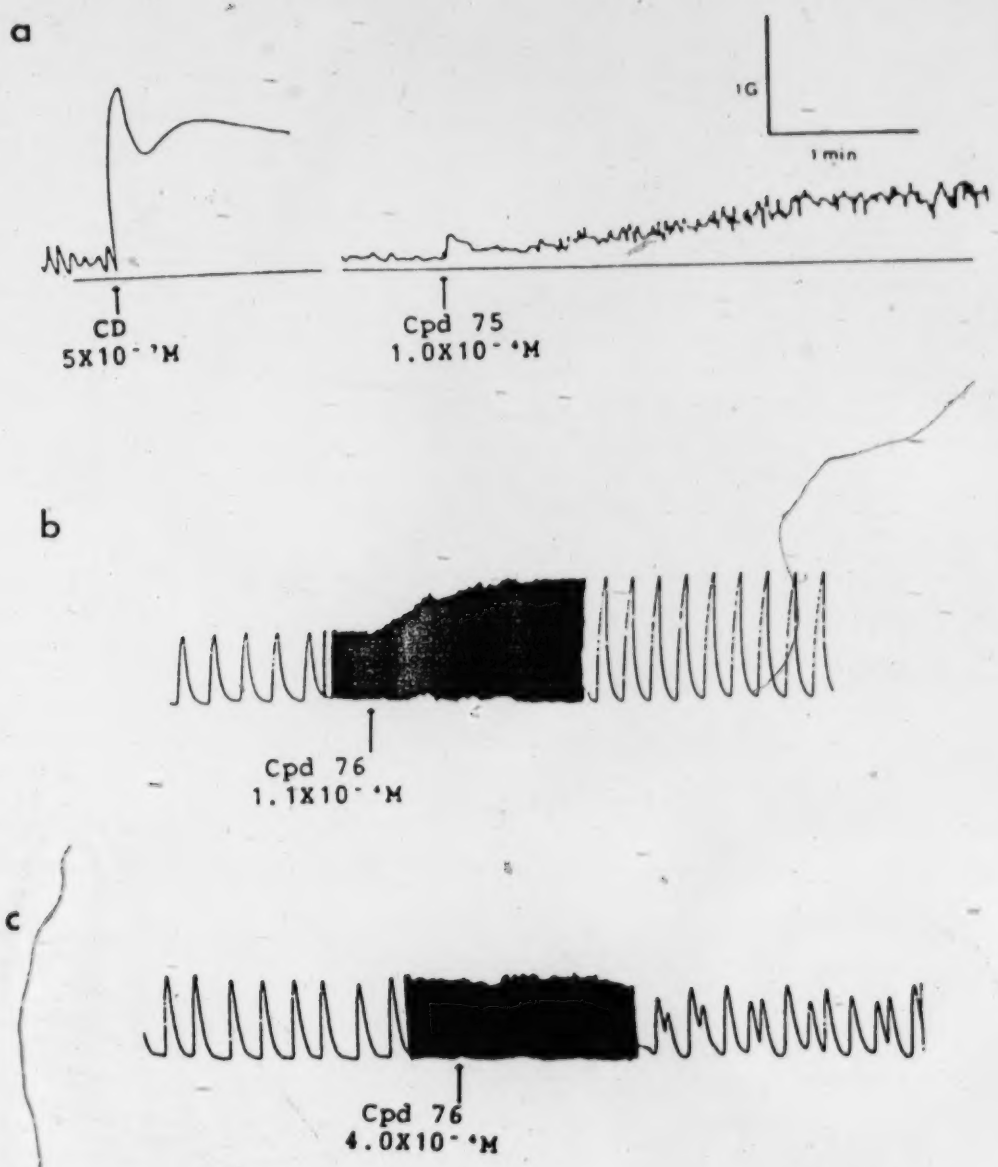


Figure 4.12 Tracing showing typical responses to the novel calcium agonists: (a) contractile activity of cpd 75 in the GPILSM (b) +ve inotropic and +ve chronotropic effects of cpd 76 in the spontaneously beating right atrium (c) cardiac disturbance caused by cpd 76 in the electrically driven left atrium.

Contractions to compounds 76 and 75 were accompanied by minute rythmical contractile activity while those to CD usually did not. The maximal contractions to compounds 76 and 75 ranged between 20 and 60 per cent of the maximal response of CD, suggesting perhaps that these compounds had a much weaker intrinsic activity than CD, and were acting as partial agonists at these concentrations, since supramaximal doses were observed to produce antagonistic effects.

Schramm and co-workers (1983) reported that the contractile activity of Bay K 8644 could only be induced in the rabbit aorta, if a priming dose of KCl was present. As depicted in Figure 4.13, dose response curves of compound 75 obtained in both the absence and presence of a priming dose of 10 mM KCl were identical. In fact it has also been reported that in other isolated preparations such as the bladder, no priming dose of KCl was necessary for the contractile activity of Bay K 8644 (Su *et al.*, 1984).

Figure 4.14 illustrates an experiment where the Ca^{2+} dependence of the responses to compound 75 were investigated. Thus no activity was recorded when a maximal dose of compound 75 was added to the calcium free HPSS. Cumulative addition of calcium then produced dose dependent contractions, suggesting that the contractile activity of compound 75 in the GPILSM is extracellular calcium dependent.

As reflected by the results in Table 4.10, the 4'-pyridinyl analog was about twice as potent as the 3'-

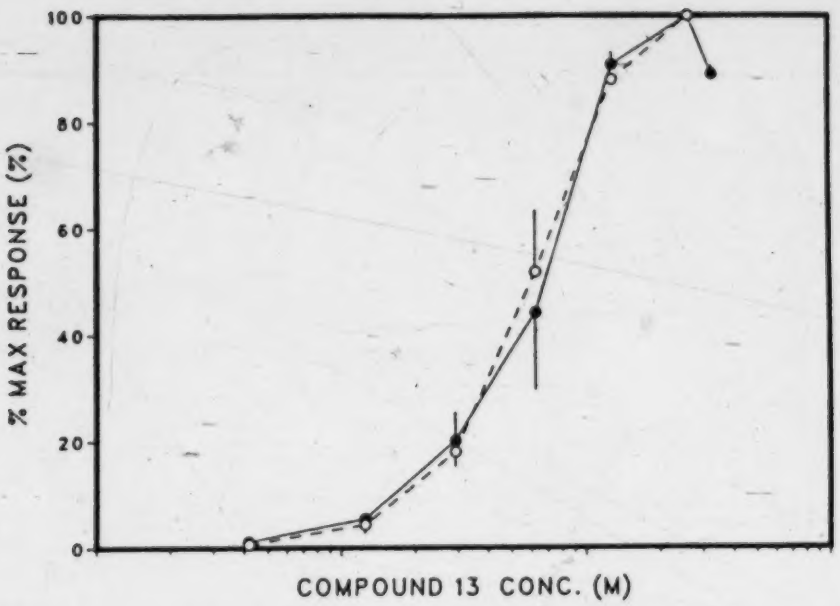


Figure 4.13 Dose-response curves of Cpd 75 with (o) and without (•) the presence of 10 mM KCl. Each point represents the mean of 5 experiments ± SEM. (GPILSM).

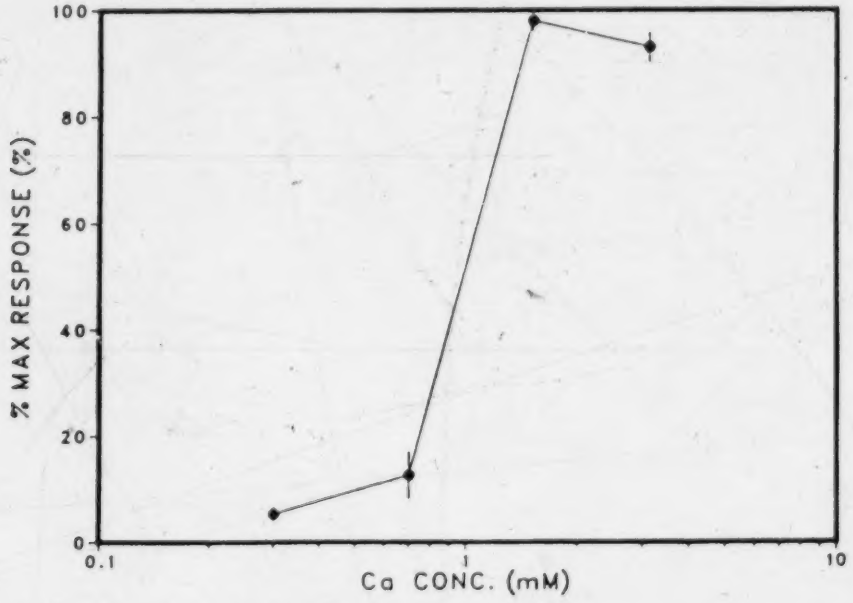


Figure 4.14 Extracellular Ca^{2+} dependence of compound 75 responses in GPILSM. Each point represents the mean of 6 experiments ± SEM.

analog in contracting the GPILSM as well as in increasing the contractile force of left atria; however both compounds were equieffective in producing +ve chronotropic responses on right atria. On the other hand, the 2'-pyridinyl analog had -ve chronotropic effect on right atria while it was virtually without effect on left atria. These results suggest that the calcium antagonist binding sites for inotropic activity in left atria and chronotropic activity in the right atria, are not similar and therefore possess different activity requirements.

Recently Lang and Triggle (1985) examined the crystal and molecular structures of Bay K8644, CGP 28392 and other dihydropyridine compounds by X-ray diffraction analysis. They reported that the calcium agonists also adopted a similar conformation to the calcium channel antagonists where the C4-substituted phenyl is perpendicularly positioned above the boat shaped 1,4-dihydropyridine ring. They went on to suggest that differences in hydrogen bonding activity at the amine group and ester substituent orientation might dictate agonist/antagonist activity of these compounds.

In the antagonist series of compounds, it was seen that the ortho-substituted phenyl ring of nifedipine was partially bioisosteric with a pyridinyl ring, such that the order of activity was 2'- > 3'- > 4'-pyridinyl analogs which is in parallel with ortho- > meta- > para-substituted phenyl compounds. However replacing the ortho-trifluoromethylphenyl

ring of Bay K8644 produced the 2'-pyridinyl calcium antagonist analog, whereas the 3'- and 4'-pyridinyl derivatives showed calcium agonist properties. Therefore it appears that the presence of a NO_2 substituent at the C3 position does not solely dictate agonist activity. However since Hof and co-workers (1985) had shown that the (-) and (+) enantiomers of a novel dihydropyridine analog possess antagonist and agonist property respectively, it may be possible that the activities of compounds 74, 75 and 76 reported in this present investigation represent the sum of the diametrical actions of the enantiomers. Hence the separate activities of the enantiomers should be evaluated.

5. CONCLUSION

5.1 General Discussion

In the present study, 76 novel dihydropyridine analogs of nifedipine were screened on the GPILSM, and 15 of these were further tested on the GP isolated atria. From the test results obtained, 74 of these compounds were found to possess calcium channel antagonistic properties while the other two compounds had calcium agonist activity. 21 of these 74 novel calcium channel blockers were evaluated on the GPILSM for their inhibitory effect on both the muscarinic and KCl-depolarization calcium dependent contractions.

The SAR derived in this study are of particular interest since this may provide further insight on the nature of the receptors to which the dihydropyridine calcium channel blockers interact. Although some of the test drugs had major structural differences as compared to nifedipine, all possessed activity. The GPILSM relaxant activity of these compounds ranged from 10^{-4}M to 10^{-8}M. If all these drugs mediate their observed pharmacological actions through the same receptor system, then it appears that these receptors are quite flexible since they can tolerate such varied structural changes. In some cases, where high concentrations were required ,such as 10^{-4}M to 10^{-5}M, a non specific action may also be possible. Overall it appears that the dihydropyridine receptors can make conformational

adjustments to interact with structurally different dihydropyridine test drugs (in parallel with conformational changes of calmodulin induced by Ca^{2+} binding), so that a "best fit" is obtained for each drug.

In the present study, the test compounds were divided into five series of analogs and their SAR were derived for each series. On considering the SAR information obtained, structural activity requirements for the dihydropyridine analogs as a whole could be generalised. Thus at the C4 position of the dihydropyridine ring of nifedipine, a flat substituted ring system was observed to be optimal as opposed to a more puckered dihydropyridinyl or an even more twisted tetrahydropyridinyl ring. This C4-ring system should preferably be perpendicularly positioned to the plane of the 1,4-dihydropyridine ring, as opposed to the co-planar orientation that exists in the 1,2-dihydropyridine or C4-2'-tetrahydropyridinyl series of compounds.

In the C4-dihydropyridinyl series, it was seen that a small substituent such as COOMe on the C4-ring yielded more active compounds than COOPh or COOtBu substituents. It however also appears that this substituent should possess a certain optimal degree of steric influence since the smaller lone pair of electrons in the pyridinyl series of compounds was not as good as the NO_2 substituent on the phenyl ring of nifedipine.

In some cases, it was observed that the SAR obtained from one series of compounds was different from that of

another series. Thus while it was established that in the C4-pyridinyl and C4-dihydropyridinyl series, an increasing order of activity was achieved through the sequence of ortho > meta > para-substituted ring; in the 1,2-dihydropyridine series the following activity sequence was obtained, para > meta > ortho. Also while in the pyridinyl series, increasing the size of the ester substituents at both C3 and C5 positions enhanced activity, the activity in the dihydropyridinyl series was only increased up to an optimal size of the ester groups and any further increase had a detrimental effect. A possible explanation of this phenomenon is that a change in one part of the drug molecule could indirectly affect the interaction of other functional groups of that molecule to the receptor. For example in the latter case mentioned, it is possible that the C4-planar pyridinyl ring interacts with a complimentary flat binding site of the receptor, and in this position the ester substituents at the C3 and C5 positions are in a favourable place to interact with their binding sites. Replacing the ideal planar ring by a more puckered dihydropyridinyl ring would offer less interaction or "fit" to that flat binding site, and through steric hindrance this could therefore affect the C3 and C5 ester substituents' orientation towards the receptor; hence a different SAR with regard to the ester substituents.

All the pharmacological evaluations of the novel compounds tested in this thesis have been presented as inhibitory effects (ID_{50} on muscarinic or KCl induced contractions). In

order to confirm that this data reasonably reflected an interaction with receptor specific sites on the calcium channel, selected samples within each series were independently assessed for their ability to displace [^{3}H]-nitrendipine from the dihydropyridine binding sites in GPILSM.

The ligand binding work was undertaken by Dr. C. R. Triggle' s research group in the Department of Pharmacology, Memorial University, St. John' s, Newfoundland, using the methods described by Bolger *et al.* (1983). Bolger and co-workers (1983) have characterised the [^{3}H]-nitrendipine binding in the GPILSM and have shown that the binding site identified, represented a specific site to which the 1,4-dihydropyridine class of calcium channel blockers interact to mediate their pharmacological action.

Table 5.1 presents the results of the binding studies and compares the previously determined ID_{50} values with the respective K values obtained. In the majority of cases there appeared to be a good correlation (1:1) between the GPILSM relaxant activity and the corresponding radioligand dissociation constants. However a large discrepancy was observed for compound 66 whose pharmacological activity was 65.8 fold more sensitive than its binding capacity. This discrepancy is interesting since it suggests that this compound probably mediates its inhibitory effect by a different mechanism which is less dependent on the dihydropyridine binding site.

Table 5.1

Comparison of GPILSM relaxant activity ID_{50} with its dissociation constant K_D obtained from radioligand binding studies.

Cpd	ID_{50}	K_D	Ratio
4	9.36×10^{-8}M	8.0×10^{-8}M	0.85
6	4.10×10^{-8}M	2.5×10^{-8}M	0.61
12	1.40×10^{-7}M	8.5×10^{-8}M	0.6
21	5.80×10^{-7}M	1.0×10^{-7}M	0.17
27	5.89×10^{-7}M	2.6×10^{-6}M	4.4
33	3.69×10^{-6}M	5.2×10^{-5}M	14
35	1.67×10^{-6}M	2.5×10^{-6}M	1.5
39	5.80×10^{-6}M	1.8×10^{-5}M	3.1
55	3.43×10^{-6}M	5.1×10^{-5}M	14.8
56	1.10×10^{-6}M	3.1×10^{-6}M	2.8
57	6.47×10^{-5}M	4.4×10^{-5}M	0.68
59	1.81×10^{-6}M	$> 10^{-4}$M	
60	4.96×10^{-6}M	8.2×10^{-5}M	16.5
63	1.20×10^{-5}M	3.0×10^{-5}M	2.5
66	7.90×10^{-6}M	5.2×10^{-4}M	65.8
67	2.52×10^{-5}M	1.5×10^{-4}M	6

Therefore the above results indicate that these novel dihydropyridine compounds mediate their action through a common [^{3}H]-nitrendipine binding site and have a common mechanism of action.

5.2 Future Considerations

Several aspects discussed in this thesis merit to be further investigated. SAR of these novel calcium channel blockers were found to be similar for both the GPILSM relaxant activity and the GP isolated atria' s chronotropic activity (however the novel calcium channel blockers were less potent in the cardiac preparations). Such SAR comparison could also be undertaken for other organ systems, in particular the vascular system. Information gathered from these studies could help in the understanding of how structural changes of the drug molecule relate to specificity for certain tissues (e.g cerebral blood vessels). It might also be possible to distinguish between the structural requirements for increasing a particular activity and for achieving specificity for a particular tissue.

In general, it was observed in this project that the phasic component of the KCl induced response was usually larger than that of the muscarinic one, whereas the muscarinic tonic response was slightly larger or equal to that of KCl. However only the calcium dependent tonic contractions were monitored in this present study. This work

could be extended to include an assessment of the effect of these novel calcium channel blockers on the phasic response of both KCl and muscarinic agonists. Such work may shed light on the nature of the similarities and differences of the KCl and muscarinic induced contractions. The possibility exists that some of the novel calcium channel blockers may be more specific for a certain type of response.

As mentioned in the Literature Survey section, Spedding (1985) subgrouped the calcium antagonists according to their lipophilicities, and dihydropyridine analogs as a whole were classified as Group I. It would therefore be interesting to determine the lipophilicity properties of these novel calcium channel blockers to see if they conform to Spedding 's hypothesis. A comparison of the lipophilicity characteristics of these compounds with their pharmacological effects would be required. Information derived from such a study, may indicate to what degree lipophilicity plays a role in the determination of the dihydropyridine calcium antagonists' SAR.

In the C4-dihydropyridinyl series of compounds, it was found that an optimum size of ester substituents at the C3 and C5 positions appear to exist, since any further increase in size reduced activity. These compounds with bulky iso-butoxycarbonyl substituents were also found to have a longer "duration of action". However no such observations were recorded in the pyridinyl series. Pyridinyl analogs with ester substituents bulkier than iso-butoxycarbonyl could be

tested to see whether the same trend exists in this series or whether it is only restricted to the dihydropyridinyl series.

It would also be worthwhile to further characterise the action of the long acting compounds. The nature of their interaction with the dihydropyridine binding site in competition with [^{3}H]-nitrendipine would be useful. If radiolabelled compounds could be designed to irreversibly interact with the calcium channel, further isolation and biochemical characterization of the channel would be feasible. If the interactions could be specific and selective such compounds may also be clinically useful for diagnostic imaging.

SAR of the calcium agonist series certainly deserves further investigation. One question that should be addressed is why replacement of the o-CF_3-phenyl ring of Bay K8644 by 2'-pyridinyl resulted in an analog which had calcium antagonistic properties while the 3'- and 4'-pyridinyl derivatives had calcium agonist characteristics. By obtaining a clear understanding of the structural features which are uniquely agonist as opposed to antagonist properties, would provide the attractive possibility for predictive designing of specific agonist/antagonist compounds.

Finally the search and identification of specific natural ligands with agonist and antagonist activities remains to be done.

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